# INDIVIDUAL PECTIN METHYLESTERASE ISOZYMES FOR PECTIN MODIFICATIONS TO INCREASE EMULSION STABILITY

by

#### JOSHUA SCOTT RIVNER

(Under the Direction of Louise Wicker)

#### ABSTRACT

Pectin methylesterase (PME), extracted from Valencia pulp, was fractionated into 36 kDa, 27 kDa, and 13 kDa isozymes and isozyme combinations using a series of ion exchange columns. The 36 and 27 kDa isozymes were salt independent while the 13 kDa isozymes lost 50% of activity without salt. The 27 and 13 kDa fractions were unstable to heat at 70 °C for 10 minutes while the 36 kDa fraction retained activity. Partial amino acid sequences were determined using mass spectrometry analysis.

The isozymes fractions were used to create charge modified pectin whose molecular weight remained the same as the unmodified control pectin. In all modified pectin samples, GGG/GGE and GG peaks increased in frequency and the EE peaks decreased in frequency compared to the control pectin. The calcium sensitivity test found a linear correlation between G' and degree of esterification (%DE) ( $R^2$ = 0.568). A correlation was observed between G' and GGG/GGE frequencies ( $R^2$ = 0.776). A smaller correlation was observed between G' and GG frequencies ( $R^2$ = 0.421). A correlation was observed between GGG/GGE frequencies and  $\zeta$ potential ( $R^2$ =0.693). These tests show that both %DE and the distribution of DE play a role in determining the functionality of the pectins. Larger blocks of continuous charge affected the functionality of the pectin more than small blocks of charge.

Pectins charge modified by separated pectin methelesterase (PME) isozymes were used to form complexes with whey protein to test their ability to stabilize an oil-in-water emulsion. No differences in emulsion activity (EA) measurements were observed between emulsions formulated with modified pectin samples. Initial EA stability was higher in emulsions with pectin and protein versus just pectin or just protein. Back scattering (BS) profiles of the emulsions showed the samples were subject to creaming, phase separation, and coalescence or flocculation.

INDEX WORDS: Pectinmethylesterase, Modified pectin, Low methoxyl pectin, Degree of esterification, Charge and charge distribution, NMR, ζ-potential, Calcium sensitivity, Emulsion stability, Emulsion activity, Shelf-life study, Turbiscan back scatter profile, Pectin, protein complexes

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## DEDICATION

I would like to dedicate this dissertation to my family for all of their positive support and love, my beautiful fiancée Marisa for her love and support, and all my friends and fellow students that helped me achieve my dream.

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### CHAPTER 1

#### INTRODUCTION

Pectin is targeted as a natural ingredient that could potential replace synthetic emulsifiers in the food industry (Garti 1999). Pectins are ionically charged structural plant polysaccharides found in the cell wall of many plants and are commonly used in the food industry as gelling, thickening, and stabilizing agents(May 1990; Savary, Hotchkiss et al. 2003). Pectin methylesterase (PME, E.C.3.1.1.11) catalyses the demethoxylation of esterified pectins. The degree of esterification (% DE) at the C6 position on the pectin chain classifies pectin between low-methoxyl (LM) (25-50% DE) and high-methoxyl (HM) (50-80% DE). Plant PME deesterify HM pectin yielding LM pectin with block-structures of charge on the pectin backbone which allows Ca<sup>++</sup> cross linking of pectin chains of LMP(Hotchkiss, Savary et al. 2002).

In citrus, multiple isozymes of PME are present. Initially there were three isozymes described in orange with one featuring a high molecular weights and thermal stability(Versteeg, Rombouts et al. 1980). Separating these differing isozymes is important because they most likely have differing action patterns and therefore effect pectin different(Kim, Teng et al. 2005). Distribution of the methyl esters along the complex pectin structure plays a key role in determining the functionalities of the pectin(De Vries, Hansen et al. 1986).

Based on the hypothesis that PME fraction containing different isozymes (36, 27, or 13 kDa) would yield differently modified pectin, the overall objectives of this study were to modify commercial pectin using separated PME isozymes to create tailored LMP to stabilize emulsions with whey protein. The third chapter describes how the various PME isozymes were separated

in solution using ion exchange chromatography (IEX) and how the isozyme fractions were characterized based on sequence, heat stability, salt dependence, and action pattern. The fourth chapter details how the isozyme fractions were used to create modified pectins with a target %DE. The chapter also depicts how the pectins were analyzed for charge and charge distribution, calcium sensitivity, and  $\zeta$ -potential or surface charge. The calcium sensitivity of the pectins was tested by the determination of the viscoelastic properties (G' and G'') on calcium gels formed by the modified pectins. The fifth chapter relates how the modified pectins were used to create emulsions with whey protein to increase the stability of the emulsion system. The emulsions were evaluated by emulsion stability, emulsion activity, particle size and shelf-life studies.

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### CHAPTER 2

#### LITERATURE REVIEW

#### 1. Pectins

Pectins are ionically charged structural plant polysaccharides found in the cell wall of many plants and are commonly used in the food industry as gelling, thickening, and stabilizing agents. Pectin is made up of three major pectic polysaccharides that all contain galacturonic acid (GalA): 1) homogalacturonan (HG), a linear polymer high in GalA content; 2) rhamnogalacturonan I (RGI), a repeating disaccharide with a mixture of glycan chains; 3) rhamnogalacturonan II (RGI) which features GalA resides and numerous structurally different oligosaccharides side chains all linked to the RGII backbone; (Ridley, O'Neill et al. 2001). The side chains contain primarily L-arabinose, D-galactose, and D-xylose (De Vries, Rombouts et al. 1982; De Vries, den Uijl et al. 1983). According to the International Pectin Producers Association, pectin is required to contain at least 65% GalA to be labeled as pectin(IPPA 2001). The homogalacturonan portions of the pectin are often identified as 'smooth' regions, while the rhamnose-rich zones are referred to as 'hairy' regions. The GalA residues that make up the pectin chain can be O-acetylates at HO-2 and/or HO-3 or methyl esterified at C-6(Nelson, Smit et al. 1977). The degree of esterification (% DE) at this position classifies pectin between lowmethoxyl (25-50% DE) and high-methoxyl (50-80% DE). Vincken, Schols et al. (2003) proposed another view of the pectin structure in which HG would be a side-chain of RG-I.

The industrial application of pectin is largely determined by the methyl-esterification of HG. The properties and biological functions of HGs are thought to be determined by ionic

interactions (Ridley, O'Neill et al. 2001; Willats, McCartney et al. 2001). Daas, Meyer-Handsem, Schols, De Ruiter, & Voragen (1999) first presented the term 'degree of blockiness' as the total amount of liberated GalA residues expressed as the proportion of the total number of free GalA residues percent in the pectin. Both the %DE and the distribution of the methyl groups or degree of blockiness have a large influence on the properties of pectin. For example, a block-wise arrangement of free carboxyl groups leads to improved calcium-gelling properties when compared to pectins with a random distribution of free carboxyl groups(Thibault and Rinaudo 1985; Ralet, Dronnet et al. 2001).

Pectin gelation occurs in LMP when calcium bridges form between two carboxyl groups in two differing chains in close contact(Axelos and Thibault 1991; Voragen, Pilnik et al. 1995). The pattern of esterification, block-wise or random, impacts the aggregation and association properties of the pectin towards cations such as calcium (Powell, Morris et al. 1982; Willats, McCartney et al. 2001). Modifying the charge and charge density of a pectin effects the gelling properties(De Vries, Hansen et al. 1986; Willats, Orfila et al. 2001) and calcium binding capability(Limberg, Korner et al. 2000). Pectin gelation properties are impacted by the length of their side branches and degree of acetylation(Matthew, Howson et al. 1990). Additionally, the charge of the pectin also affects the ability of the pectin to act as a stabilizer in low pH environments (Glahn 1982).

Pectin's highly complex structure varies based on its source and extraction process(Akhtar, Dickinson et al. 2002). The distribution of the methyl esters along the complex pectin structure plays a key role in determining the functionalities of the pectin(De Vries, Hansen et al. 1986). Finding an ideal 'designer pectin' is the goal of much of the current research as

modifying the structure of the pectin with chemical, enzymatic, or physical manipulation can lead to higher specific functionalities(Willats, Knox et al. 2006).

#### 2. Pectin Methylesterase

Pectin methylesterase (PME, E.C..3.1.1.11) catalyses the demethoxylation of esterified pectins. When PME catalyzes pectin, the demethylation of pectin occurs leaving blocks of deesterfied pectin freeing hydrogen ions and methanol. PME also plays important roles in cell processes such as plant response to pathogen attack(Markovi and Jornvall 1986; Dorokhov, Mäkinen et al. 1999), management of cell wall modifications during fruit ripening(Pressey 1984; Micheli 2001), cell growth and extensions(Nari, Noat et al. 1991), and control of pollen tube growth(Mu, Stains et al. 1994; Bosch, Cheung et al. 2005).

PME can be extracted from several varying sources (plants, bacteria, and fungi) and the differing sources yield different action patterns in regards to the elimination of methyl esters. Furthermore, in Valencia orange PME, different individual isozymes in the PME can be characterized by expression patterns, physical and biochemical properties(Bordenave 1996; Savary, Hotchkiss et al. 2002).

In citrus, multiple isozymes of PME are present. Initially there were three isozymes described in orange with one featuring a high molecular weights and thermal stability(Versteeg, Rombouts et al. 1980). Another lab group purified and partially sequenced a putative thermostable PME from citrus fruit (Nairn, Lewandowski et al. 1998; Arias and Burns 2002).

There are three proteins that Wicker's lab has identified with PME activity in citrus fruit at 36, 27, and 13 kDa(Kim, Teng et al. 2005). In one study, partially purified PME that contained proteins at 36 kDa and 13 kDa or 36 kDa and 27 kDa were compared. That study found that while both fraction modified pectins in a blockwise manner, the PME fraction that

contained 36 kDa and 27 kDa peptides left larger blocks of continuous charge than the fraction that contained the 36 kDa and 13 kDa peptides(Kim and Wicker 2006). They also found that the two PME fractions differed in the amount of intramaolecular de-esterification, amount of molecules modified, and the gelling ability(Kim, Teng et al. 2005).

Another group of studies detailed four PME isoforms in Valencia differing in molecular weight, salt dependency, pH dependency and thermostability (Hotchkiss, Savary et al. 2002; Savary, Hotchkiss et al. 2002; Cameron, Savary et al. 2003; Savary, Hotchkiss et al. 2003). The PME fractions were separated into one peak containing 34/8 as salt independent(Savary, Hotchkiss et al. 2002) and a fraction that contained 34 kDa and determined it to be salt dependent (Cameron, Savary et al. 2003). They obtained a partial sequence for these proteins that contained six amino acids. They showed the sequence data for the protein at 34 kDa and not the protein at 8 kDa(Savary, Hotchkiss et al. 2002). They also sequenced a protein at 27 kDa from a commercial orange PME and asserted that it was analogous to their 34 kDa sequence(Savary, Hotchkiss et al. 2002). Separating these differing isozymes is important because they most likely have differing action patterns and therefore effect pectin differently(Kim, Teng et al. 2005). The combination of 36 and 27 or 36 and 13 PME may modify pectin differently by means of a combination effect, meaning that the combination of multiple PME isozymes may cause a greater amount of modification than just an individual PME alone. The action pattern of the individual isozymes can only be known if individual isozyme is used to modify a pectin sample. Another lab group obtained two partial amino acid sequences from PME fragments of the 36 kDa isozyme (HQAVALRV and TYLGRPWK) (Nairn, Lewandowski et al. 1998) and used reverse genetics approach to identify PME genes(Nairn, Lewandowski et al. 1998; Arias and Burns 2002).

PME isozymes have been the subject of several studies due to their negative impact on juice quality by causing cloud loss in juice and gelation in juice concentrate(Pilnik and Voragen 1991; Laratta, Fasanaro et al. 1995). PME in juice also causes the phenomenon known as clarification which is when PME causes the creation of free carboxyl groups that in the company of naturally occurring calcium ion yield pectin precipitation as calcium pectate. PME activity is also associated with many of the negative changes that occur during fruit and vegetable processing. Active PME in fruits and vegetable products yields demethylated pectins which can be hydrolyzed by polygalacturonase, resulting in shorter chains and harsh changes in the final product such as a decrease in firmness and reduced viscosity (Castaldo, Lovoi et al. 1991; Laratta, Fasanaro et al. 1995; Castaldo, Laratta et al. 1997). In light of these PME characteristics, controlling PME activity through temperature and saline concentration is important in the food industry for producing a higher quality product.

#### **3.** Action Pattern Analysis

The three main action patterns (which were originally developed to describe starch but apply to pectin) are: 1) the multiple chain mechanism, where dissociation of the enzymesubstrate complex after the reaction results in deesterification of merely one residue per attack; 2) the single chain mechanism, where the enzyme binding is followed by a conversion of all not contiguous substrate sites on the homogalacturonan; 3)the multiple attack mechanism, where every active enzyme complex produced results in the enzyme catalysis of the deesterification of a limited number of residues(Grenwood and Milne 1968).

The various action patterns of the PME isozymes are important because the pattern affects the charge distribution on the pectin. PME from higher plants (such as banana, tomato, apple, orange, strawberry) tends to catalyze the demethylation of pectin using the single chain

mechanism which results in a block-wise arrangement of free carboxyl groups in the pectin molecules. The de-esterification process results in the introduction of block-structure of adjoining free galacturonic acid units on the HG backbone allowing for calcium cross-linking of pectin chains(Thibault and Rinaudo 1985; Christensen, Nielsen et al. 1998; Limberg, Korner et al. 2000; Savary, Hotchkiss et al. 2002; Wicker, Ackerley et al. 2003)

There have been several methods used to reveal the variations in action patterns of differing PME. The blocks of charge on pectins have been analyzed by enzymatic methods employing exo-polygalacturonase (exo-PG) and endo-polygalacturonase (EPG) (Limberg, Körner et al. 2000). Pectin lyase, which cleaves the HG in high methylated regions, was used to estimate lengths of partially esterfield blocks by Limberg, Korner, et al. (2000). Distinction between pectins modified with plant PME (block-wise demethylation) and fungal PME or base catalysis (random demethylation) in respect to the action pattern of the demethylation have been shown with enzymatic fingerprinting with EPG(Daas, Voragen et al. 2001) and EPG II, as well as methods using specific pectin antibodies(Willats, Limberg et al. 2000). EPG has also been used to study action patterns by looking for blocks of charge on the pectins(Daas, Meyer-Hansen et al. 1999). Capillary zone electrophoresis was utilized to analyze %DE and found there was a correlation between % DE and migration times of pectin (the higher the %DE, the shorter the migration time(Jiang, Liu et al. 2005). Zhong, Williams, Keenan, Goodall & Rolin (1997) also found a linear relationship between %DE and migration time and in a second study Zhong, Williams, Goodall, and Hansen(1998) developed a method to interpret capillary electrophoresis peaks to determine distribution of %DE.

Nuclear magnetic resonance (NMR) studies have revealed variations in action patterns of these differing PME isozymes combinations (Grasdalen, Andersen et al. 1996; Kim, Teng et al.

2005). Indirect statistical methods on GalA oligomers with differing %DE's measured with NMR have been used to measure the blocks of charge on pectin(Catoire, Pierron et al. 1998; Denes, Baron et al. 2000). Colquhoun, de Ruiter, Schols and Voragen (1990) used 2D- NMR to analyze the oligosaccharides on the hairy region of modified apple pectin. A recent study utilized Nuclear Overhauser effect spectroscopy NMR to predict degree of random de-esterification and degree of block de-esterification. The study showed NMR was a good tool for this prediction, though the researchers had greater success with random de-esterification than block-wise(Winning, Viereck et al. 2007).

<sup>1</sup>H NMR is also a valuable tool for determining the %DE of pectin. One can calculate the %DE from NMR by using the chemical shifts of protons on E (esterified galacturonic acid) and G (de-esterified galacturonic acid) at H-4(Grasdalen, Andersen et al. 1996). NMR % DE analysis is advantageous because there is no need for external standards and requires a small amount of pectin sample.

### 4. Rheology

Pectins are often modified to affect their gelling activity. The gelling activity of pectin is important in the industry for creating gels and its ability as a stabilizer in emulsions. Pectin gelation occurs in LMP,DE's between 35-50%, when calcium bridges form between two carboxyl groups in two differing chains in close contact(Axelos and Thibault 1991; Voragen, Pilnik et al. 1995). LMP gels in the presence of cations such as calcium. HMP with DE's above 50%, relies on acidic pH, sucrose concentration, and the pectin's ability to form hydrogen and hydrophobic interactions (Voragen, Pilnik et al. 1995). Modifying the charge and charge density of a pectin effects the gelling properties(Willats, Orfila et al. 2001) and calcium binding capability(Limberg, Korner et al. 2000).

The gel properties of pectin are greatly dependent on the molecular properties of the carbohydrate. Pectin exhibits a negative charge in slightly acidic solutions due to the existence of ionized carboxylic groups along the pectin backbone that have a pK<sub>a</sub> value around 3.5(Plaschina, Braudo et al. 1978). This negative charge causes the pectin to interact with positively charged protein forming an interfacial membrane that stabilizes the emulsion. The charge of the pectin also affects the ability of the pectin to act as a stabilizer in low pH environments (Glahn 1982). Manipulating the charge or charge distribution on the pectin can affect the pectin's ability to stabilize emulsions. For instance, the gelation properties of a sugar beet pectin are related to the size of the side chains and the degree of acetylation(Matthew, Howson et al. 1990). The network and aggregation properties of pectin are influenced by the pattern of esterification i.e. block-wise or random(Powell, Morris et al. 1982).

Gel strength is commonly evaluated by measuring the storage and loss moduli, G' and G''. G', the storage modulus, is defined as the ratio of stress in phase with the strain to the strain. G'', the loss modulus, is defined as the ratio of the shear stress  $90^{\circ}$  out of phase with the strain to the strain (Bourne 2002). In other words, G' is a measurement of the energy stored in the gel while G'' is the apparent viscous section in the gel. Strong gels characteristically feature G' values much larger than their G'' values.

#### 5. ζ- Potential of Pectin Dispersions

Resolving the surface potential of a pectin sample can be challenging. A good way to approximate this value is to use  $\zeta$ -potential.  $\zeta$ -potential is a measurement of the magnitude of the repulsion or attraction between particles, and this measurement sheds lights on the dispersion mechanism and is crucial for electrostatic dispersion control (Farinato and Dubin 1999).  $\zeta$ -potential is a valuable particle parameter description and can be used to judge the stability of a

colloidal system(Anema and Klostermeyer 1996; Farinato and Dubin 1999). The  $\zeta$ -potential measurement allows us to use the predictive method of emulsion stability(Mengual, Meunier et al. 1999). In other words, the larger the negative charge on the hydrocolloid, the more the charge will impede upon coalescence and increase stability (Morrison and Ross 2002). Some commonly used methods of determining  $\zeta$ -potential include electrophoretic light scattering (Doppler shifting of scattered light due to particle motion in a direct current electric field), electrophoresis (particle motion in a direct current electric field), electroosmosis, sedimentation potential, streaming potential, and diffusiophoresis (Hidalgo-Álvarez, Martín et al. 1996; Farinato and Dubin 1999).

A study measured the  $\zeta$ -potential of soybean soluble polysaccharide (SSPS) which has a structure similar to pectin, pectin, and pectin digested by enzymes at pH 2-7 (Nakamura, Furuta et al. 2003). The researchers found that the negative  $\zeta$ -potential of SSPS was smaller than that of pectin. The enzyme treated pectin had amplified negative  $\zeta$ -potential over control pectin because the galacturonic acids which were not methylesterified were digested and lost from the main pectin backbone. This is consistent with observations made by Kim & Wicker (2007) who reported that enzyme modified pectins had higher negative  $\zeta$ -potential values than non treated pectin. However, Kim and Wicker(2007) did not observe this trend in pectins separated by IEX chromatography making a case that  $\zeta$ -potential is hard to interpret. Kulmyrzaev and others (2000) suggested that various minerals bind to oppositely charged groups on the surface of emulsion droplets which results in a decrease  $\zeta$ -potential and reducing the electrostatic repulsion between the droplet.

#### 6. Molecular Weight

Molecular weight is another important property of pectin that can be manipulated to affect the structure of pectin. The typical pectin molecule features a molecular mass of approximately 1000 kDa(Voragen, Pilnik et al. 1995; Perez, Mazeau et al. 2000; Daas, Voragen et al. 2001). Akhtar, Dickinson et al (2002) found that a low molecular weight citrus pectin (60-70 kg mol) and a high degree of methoxylation had the most desirable emulsifying properties(Akhtar, Dickinson et al. 2002).

There have been several studies that have looked at molecular weight in batches of similarly prepared pectin. Pectins tend to feature a great deal of heterogeneity in the molecular weight due to the hairy and smooth regions of pectin and the various inter- and intramolecular distribution of methyl esters. Pectin modified by salt-independent orange PME that featured a protein at 36 kDa and 8 kDa demonstrated no significant difference in molecular weight to the original unmodified pectin (Hotchkiss, Savary et al. 2002; Kim, Teng et al. 2005) . Hellin et al.(2005)(Hellin, Ralet et al. 2005) were able to produce a series of "homogenous" lime pectin fractions all having the same molar mass and charge density purified by size exclusion and anion exchange chromatography. This was accomplished by degrading pectin with a rhamnogalacturonan hydrolase that degrades the pectin side chains(Hellin, Ralet et al. 2005).

#### 7. Emulsions

Emulsion stability is an important hurdle to overcome to ensure formulated food quality. Single emulsions feature two immiscible liquids, for example water and oil, dispersed in each other(Ficheux, Bonakdar et al. 1998). Coalescence embodies the general idea of single emulsion metastability and is the guiding principles behind the rules of stability. Coalescence is when the thin film that forms between two neighboring droplets in the emulsion ruptures due to a small

hole that forms in the thin film resulting in the synthesis of the two droplets(Ficheux, Bonakdar et al. 1998). A stable emulsion features droplets that are barred from joining together by electrostatic or steric stabilizations. The droplet surface must be fully covered or bridging flocculation will occur(Benichou, Aserin et al. 2002).

Proteins and polysaccharides impact the structure formation and stability of many food systems. Traditionally, proteins are used in the industry as emulsifying agents while polysaccharides are used as stabilizing agents. Studies of their interactions tend to focus on bestowing optimal structural quality to the formulation of new foods(Beaulieu, Turgeon et al. 2001). Mixtures of both biopolymers tend to cause a synergistic effect. The covalent proteinpolysaccharide complexion enhance stability by forming a thicker, stronger steric-stabilizing layer around the droplets than just a protein alone would(Tokaev, Gurov et al. 1987; Benichou, Aserin et al. 2002). The overall stability of the mixture depends not only on the functional properties of the distinct components, but also on the features and potency of the proteinpolysaccharide interactions(Dickinson 1995). The attractive forces between the biopolymers results in soluble complexes. Conversely, if the biopolymers bear similar charges, the complex will be thermodynamically opposing and can lead to segregative phase separation if the polymer concentration is larger than the cosolubility limit(de Kruif and Tuinier 2001). In an emulsion system that includes both a protein and a polysaccharide, the proteins usually build an absorbed layer at the water and oil interface while the hydrophilic polysaccharides forms a thick gel like secondary layer that augments the steric stabilizing properties of the protein coated droplets (Tokaev, Gurov et al. 1987; Dickinson, Elverson et al. 1989; Dickinson 1994; Xie and Hettiarachchy 1997; McClements 2004).

Pectin has been shown to be a strong emulsifier because of its ability to ease formation, increase stability, and yield favorable physicochemical properties in oil-in-water (o/w) emulsions(Akhtar, Dickinson et al. 2002; Dickinson 2003; McClements 2004). Additionally, pectin is commonly used to stabilize acidified milk drinks(Tromp, de Kruif et al. 2004). This stabilization is accomplished through pectin reacting with casein and preventing the aggregation of the protein at pH below the isoelectric point (IEP) of casein (pH 4.6). Many common acidified drink products are formulated at pH 4.2 (Tromp, de Kruif et al. 2004). Furthermore, at pH above the pI of the protein, complexes do not form with globular proteins(Tolstogusov 1986). Einhorn-Stoll, Glasenapp, & Kunzek (1996) found that pectin and protein complexes produced greater stabilization at pH 4 than at pH 7. Casein adsorbs to pectin at pH below 5.0 showing that the interaction is primarily electrostatic(Tuinier, Rolin et al. 2002).

Another study found that interactions between both low and high methoxyl pectin and  $\beta$ lactoglobulin to be largely caused by electrostatic interactions(Girard, Turgeon et al. 2002). Einhorn, Glasenapp & Kunzek(1996) reported that the lower %DE in LMP, the more pectin whey protein complexes could be formed improving emulsion stability. Several studies have reported improved emulsifying capacity when whey protein is used with a polysaccharide than just the use of whey protein alone(Hattori, Aiba et al. 1996; Mishra, Mann et al. 2001). Girard and others (2002) found that  $\beta$ -lg formed more complexes with LMP then with HMP at pH 4.5.

Based on the hypothesis that PME fraction containing different isozymes (36, 27, or 13 kDa) would yield differently modified pectin, the overall objectives of this study were to modify commercial pectin using separated PME isozymes to create tailored LMP to stabilize emulsions with whey protein. In the third chapter, the various PME isozymes were separated in solution using ion exchange chromatography (IEX) and the isozyme fractions were characterized based

on sequence, heat stability, salt dependence, and action pattern. In the fourth chapter, the isozyme fractions were used to create modified pectins with a target %DE. The pectins were analyzed for charge and charge distribution, calcium sensitivity, and  $\zeta$ -potential or surface charge. The calcium sensitivity was tested by the determination of the viscoelastic properties (G' and G'') on calcium gels formed by the modified pectins. In the fifth chapter, the modified pectins were used to create emulsions with whey protein to increase the stability of the emulsion system. The emulsions were evaluated by emulsion stability, emulsion activity, particle size and shelf-life studies.

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## **CHAPTER 3**

# CHARACTERIZATION OF SEPARATED CITRUS PME ISOZYMES THROUGH HEAT STABILITY, SALT DEPENDENCY, PROTEOMICS, AND ACTION PATTERN<sup>1</sup>

## Abstract

Pectin methylesterase (PME), extracted from Valencia pulp, was fractionated into 36 kDa, 27 kDa, and 13 kDa isozymes and combinations using a series of ion exchange columns. The 36 and 27 kDa isozymes were salt independent while the 13 kDa isozymes lost 50% of activity without salt. The 27 and 13 kDa fractions were unstable to heat at 70 °C for 10 minutes while the 36 kDa fraction retained activity. Partial amino acid sequences were determined using mass spectrometry analysis and protolytic digests. The isozymes were used to modify pectin and the charge distribution patterns on the resulting modified pectin were analyzed. The isozymes produce charged modified pectin with differing distribution patterns of charge along the pectin backbone. 36 kDa isozyme left the largest amount of blocks of continuous charge. Furthermore, combinations of multiple isozymes left larger blocks of continuous charge than individual isozymes.

#### **1. Introduction**

Pectin methylesterase (PME, E.C..3.1.1.11) catalyses the demethoxylation of esterified pectins. Pectins, ionically charged structural plant polysaccharides found in the cell wall of plants, are

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commonly used in the food industry as gelling, thickening, and stabilizing agents(May 1990; Voragen, Pilnik et al. 1995). When PME catalyzes pectin, the demethylation of pectin occurs leaving blocks of deesterfied pectin freeing hydrogen ions and methanol. PME also plays important roles in cell processes such as plant response to pathogen attack(Markovi and Jornvall 1986; Dorokhov, Mäkinen et al. 1999), management of cell wall modifications during fruit ripening(Pressey 1984; Micheli 2001), cell growth and extensions(Nari, Noat et al. 1991), and control of pollen tube growth(Mu, Stains et al. 1994; Bosch, Cheung et al. 2005).

In citrus, multiple isozymes of PME are present. Initially there were three isozymes described in orange with one featuring a high molecular weights and thermal stability(Versteeg, Rombouts et al. 1980). Another lab group purified and partially sequenced a putative thermostable PME from citrus fruit(Nairn, Lewandowski et al. 1998; Arias and Burns 2002).

A different group of studies detailed four PME isoforms in Valencia differing in molecular weight, salt dependency, pH dependency and thermostability (Hotchkiss, Savary et al. 2002; Savary, Hotchkiss et al. 2002; Cameron, Savary et al. 2003; Savary, Hotchkiss et al. 2003). The PME fractions were separated into one peak containing 34/8 as salt independent(Savary, Hotchkiss et al. 2002) and a fraction that contained 34 kDa and determined it to be salt dependent (Cameron, Savary et al. 2003). They obtained a partial sequence for these proteins that contained six amino acids. They showed the sequence data for the protein at 34 kDa and not the protein at 8 kDa(Savary, Hotchkiss et al. 2002). They also sequenced a protein at 27 kDa from a commercial orange PME and maintained it to be analogous to their 34 kDa sequence(Savary, Hotchkiss et al. 2002). Another lab group obtained two partial amino acid sequences from PME fragments of the 36 kDa isozyme (HQAVALRV and TYLGRPWK)

(Nairn, Lewandowski et al. 1998) and used reverse genetics approach to identify PME genes(Nairn, Lewandowski et al. 1998; Arias and Burns 2002).

While, nuclear magnetic resonance (NMR) and enzyme fingerprinting studies have revealed variations in action patterns of the citrus PME isozymes (Grasdalen, Andersen et al. 1996; Catoire, Pierron et al. 1998; Denes, Baron et al. 2000; Kim, Teng et al. 2005; Luzio and Cameron 2008), the 13 kDa isozyme has never been fully separated in solution. Separating these differing isozymes is important because they most likely have differing action patterns and therefore effect pectin different(Kim, Teng et al. 2005). The combination of 36 and 27 or 36 and 13 PME may modify pectin differently by means of a combination effect, meaning that the combination of multiple PME isozymes may cause a greater amount of modification than just an individual PME alone. The action pattern of the individual isozymes can only be known if individual isozyme is used on a pectin sample. Distribution of the methyl esters along the complex pectin structure plays a key role in determining the functionalities of the pectin(De Vries, Hansen et al. 1986).

The current paper details a series of ionic exchange columns to separate the various Valencia PME isozymes in solution. Once separated, the paper characterizes the individual isozymes based on salt dependency and heat stability. Partial sequences of the isozymes are obtained using mass spectrometry/ mass spectrometry (MS/MS) in order to use reverse genetics approach to indentify the PME's full sequence. High methoxyl pectin (HMP) is modified with the various isozymes to see if the various separated isozymes do have differing action patterns as predicted and if there are combination effects in enzyme featuring multiple isozymes.

#### 2. Materials and Methods

#### 2.1 Materials

Crude Valencia PME was extracted from Valencia orange pulp from 2005 processing season (donated by Citrus World, Lake Wales, Fl). Commercial high methoxyl pectin (GENU pectin type B rapid set-z, Batch G 32 691) was donated by CP Kelco (Lille Skensved, Denmark). *2.2 Valencia PME Preparation* 

The crude PME extract was created following the method described by Wicker and others (1998). Frozen pulp in a 1:5 ratio with 0.1 M NaCl, 0.25 M Tris buffer at pH 8 was homogenized on ice for 5 minutes to extract the crude enzyme. The extract was adjusted to pH 8.0 and centrifuged (Sorvall RC-5B centrifuge, DuPont Instruments, Doraville, GA) at 8000 g, 4°C for 20 minutes. Non-PME material in the crude extract was removed using a 30% ammonium sulfate precipitation. After centrifugation, the supernatant was dialyzed overnight against 0.05M sodium phosphate at pH 7.0, 4°C. The dialysis tubing (Spectra/Por, MWCO 6000, Fisher Scientific, Atlanta, GA) was prepared by boiling in 10% acetic acid and rinsing in deionized water to minimize the loss of PME activity.

To separate the PME into different fractions, an Åkta Prime system (Amersham Pharmacia Biotech Uppsala, Sweden) was used to perform chromatography following a modified method used by Wicker, Ackerley et al. (Wicker, Ackerley et al. 2003). Start buffer was 0.05 M sodium phosphate, pH 7.0 buffer and elution buffer included 1 M NaCl. Chromatography buffers were degassed and filtered through a 0.45 µm cellulose nitrate membrane filter (Whatman, Clifton, NJ) before use. Aliquots of 2 mL fractions were collected using a 50 ml linear gradient at 5 ml/min. The crude PME was loaded onto a 5 mL Hi-Trap SP cation exchange column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) at 5 ml/min. A different aliquot of PME that did bind to the Hi-Trap SP column was loaded onto a 5 ml Hi-Trap Q HP anion exchange column. The PME that did not bind to the Hi-Trap SP column was loaded onto a

5 ml Heparin (HP) affinity column (**Figure 3.1**). The collected fractions was qualitatively identified using bromthymol blue dye solution at 7.5 pH (Corredig, Kerr et al. 2000) and positive fractions were quantified by titrimetric assay. The fractions were identified as shown in **Figure 3.1**.

The activity of the isozymes were determined quantitatively using a pH stat titrator (Brinkmann, Westbury, NY) at 30° C in 1% high methoxyl pectin (GENU pectin type B rapid set-Z, Batch G 32 691, CP Kelco, Copenhagen, Denmark) and 0.1 M NaCl at a set point of pH 7.5. A unit of PME activity was defined as the microequivalent of ester hydrolyzed/min at 30° C.

Protein was quantified by the Bradford protein assay(Bradford 1976) using a Microplate Reader (MPR Model 550, Bio-Rad Inc., Hercules, CA) with IgG as the standard.

The molecular weight of the putative PME isozymes was determined by denaturing SDS PAGE on an 8-25% PhastGel gradient gel using silver stain according to manufacturer's guidelines (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). An aliquot of 0.5 µg of protein was applied to the gel for each sample. The SDS-PAGE silver stain with PhastGel media has an average sensitivity limit of 0.3 to 0.5 ng protein per band(Biosciences 1991).

## 2.3 Salt Dependency

Aliquot of 2 ml fractions were dialyzed against 500 ml of deionized water using Slide-A-Lyzer 10,000 MWCO Dialysis Cassettes (Pierce Chemical Company, Rockford, IL) at 4°C overnight in order to remove salt from the samples. There was no precipitant observed in the Slida-A-Lyzer after dialysis. The activity of individual isozymes on 1% pectin with or without 0.1 M NaCl was determined using a pH stat titrator (Brinkmann, Westerbury, NY) at 30° C and the difference in activity was compared. The salt dependency test was run in duplicate for each sample and is reported as average values.

## 2.4 Heat Stability

The isozyme samples were diluted with 0.05 M sodium phosphate, pH 7.0 buffer. 10 ml of the diluted isozyme samples were heated for ten minutes at 70°C. This time and temperature combination was chosen because it is a time and temperature used during orange juice processing in order to ensure microbial destruction. Each sample was run in duplicate and the average value of activity was reported using a pH stat titrator (Brinkmann, Westerbury, NY) at 30° C. 5 mL of heat treated enzyme per sample were added before declaring no activity in the samples.

#### 2.5 Proteomics

Proteomic research was completed at the University of Georgia Proteomics and Mass Spectrometry Facility.

## 2.5.1 Gel Processing

A modified method described by Shevchenko, Wilm, et al. (1996) was used to obtain proteolytic digests of the 36, 27, and 13 kDa peptides. An aliquot of 2 mm SDS protein band pieces were cut from the SDS-PAGE gel. The pieces were then washed twice with 50 mM ammonium bicarbonate/50% methanol at room temperature with rocking until no visible stain remained. The band pieces were washed with 1 mL 75% acetonitrile for 20 minutes at room temperature. The bands were then dried in an oven at 37°C to remove the acetonitrile. Once dry, the bands were incubated in 75  $\mu$ l of 10 mM dithiothreitol (DTT)/20 mM ammonium bicarbonate, pH 8.0 at 37°C for one hour. The DDT solution was removed using a pipette and immediately replaced with 75  $\mu$ l of 100 mM iodacetamide/ 20 mM ammonium bicarbonate and incubated at room temperature in the dark for 30 minutes. The band pieces were washed by adding the 10 mM DTT/ 20 mM ammonium bicarbonate solution at 37 °C, incubating for 30

minutes and then removing the solution. Then the bands were incubated with 200 ng sequencing grade trypsin with a specific activity of 17,000 U/mg (Promega, Madison, Wisconsin) at 37°C for 16 hours. Peptides were extracted twice with 50% acetonitrile/ 0.1% *Trifluoroacetic acid* (TFA) for 20 minutes at room temperature and concentrated by SpeedVac (Jouan, St-Herblain, France). Approximately 25% of the resulting peptides were spotted on a MALDI plate with 50% saturated  $\alpha$ -cyano-4-hydroxy-cinnamic acid (Sigma-Aldrich, St. Louis, Missouri).

## 2.5.2 MS Analysis

Mass spectrometry (MS) and MS/MS data was required following a modified version of the procedure presented by Shevchenko, Wilm, et al. (1996). MS and MS/MS data were obtained on a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, California) using standard acquisition methods (positive mode, reflectron). MS/MS data were achieved in a data dependent fashion meaning the instrument selects the top ten most intense peaks in the MS spectrum and performs MS/MS on the selected peaks. MS spectra were calibrated using two trypsin autolysis peaks (104.5 and 2211.1 m/z) to ensure internal calibration of the MS spectrum and improved mass accuracy. MS/MS spectra were calibrated using the instrument default processing method because MS/MS can handle a wider mass charge window than MS spectra alone. The most recent version of NCBInr database was incorporated into a licensed copy of Mascot 1.9.05 (http://www. Matrixscience.com/). Mass lists were submitted to Mascot (Viridiplantae taxonomy) considering fixed cysteine carbamidomethylation and partial methionine oxidation modifications, 1 missed tryptic cleavage, and 25 ppm mass accuracy. Identifications were cross-examined using mass accuracy and molecular weight. For those proteins not identified using standard correlative searching strategies, de novo sequence was deduced from MS/MS spectra using Data Explorer and Denovo Explorer software tools to aid in

manual interpretation of spectra. *De novo* sequences were submitted to MS BLAST (<u>http://dove.embl-heidelberg.de/Blast2/msblast.html</u>), and aligned to a NCBInr database of sequences.

#### 2.6 Pectin modification

A quantity of the 36, 27, or 13 PME isozyme were used to de-esterify citrus pectin to a target % degree of esterification (DE) within a specified time. In extractions 1 and 2, the target DE was 10% lower than control pectin. In extraction 3, the target DE was 20% lower than control. A pectin dispersion (1% pectin in experiments 1 and 2, 3% pectin in experiment 3) in 0.1 N NaCl was equilibrated at 30° C and adjusted to pH 7.5 with NaOH. The isozyme was added at a calculated amount to give the target %DE within approximately 35 minutes while the pH was maintained at pH 7.5 with NaOH. The PME activity in the dispersion was stopped by decreasing the pH to 4.5, adding 95% ethanol for a final concentration of 80% ethanol, and boiling for 10 minutes. The dispersion was cooled to room temperature and the modified pectin was washed four times in ethanol and once in acetone. Control pectin made with a 3% pectin solution was maintained at pH 7.5 for 35 minutes; pH was decreased to 4.5, boiled in ethanol and washed.

#### 2.7 NMR on modified pectin

The %DE and distribution of DE of the pectin was estimated by <sup>1</sup>H NMR spectroscopy in accordance to a previous study(Kim, Teng et al. 2005). The pectin samples were prepared by using 6 mg of sample pectin in 0.7 ml 50 mM phosphate buffer at pH 7.0. The sample was lyophilized five times in D<sub>2</sub>O to remove the solvent protons. On the day of sample analysis, the samples were dissolved in 0.75 ml 99.95% D<sub>2</sub>O. The NMR spectra were captured using a Varian Iova 500 Hz spectrometer (Varian, Inc., Palo Alto, CA) at 80°C. Each sample was run

four times with 64 scans on each of the four runs. The overlapped peaks were resolved and peak areas were computed using OriginPro 7.5 software (Origin Lab Co., Northampton, MA). The peak assignments were selected using the previously published assignments (Grasdalen, Andersen et al. 1996; Denes, Baron et al. 2000; Rosenbohm, Lundt et al. 2003; Kim, Teng et al. 2005). The spectra generated in this study did not have the resolution to resolve the GGG and GGE nor EEG and EGE peaks. They are instead labeled as GGG/GGE and EEG/ EGE respectively, as shown in **Figure 3.2**. The values of the dyads and triads fractions, as well as the value of %DE, were calculated using the integration volumes of the assigned spectral peaks based on the relationships(Grasdalen, Andersen et al. 1996):

 $DE = I_E(H-4) / I_E(H-4) + I_G(H-4)$ 

 $F_{GGG} = I_{GGG}(H-5)/I_{E}(H-4) + I_{G}(H-4)$ 

Where "I" stands for the integration volumes, "E" represents esterified resonances and "G" corresponds to de-esterified resonances.

## **3.0 Results and Discussion**

#### 3.1 PME isozyme separation

PME isozymes of 36, 27, and 13 kDa were successfully separated into the individual peptides using a series of ion exchange columns. The separation of all Valencia orange PME isozymes has not been previously accomplished before in the literature. The specific columns, order of columns, and the resulting separation are shown in **Figure 3.1**. Notice that extraction 1 differed from extraction 2 and 3 by employing a Heparin cation exchange column after the SP column instead of a Q anion exchange column. There was a yellow color associated with the PME fractions that had activity and the more PME U/ml the fraction contained, the darker the yellow color.

The PME fractions shown in **Figure 3.1** show that the 13 kDa isozyme binds strongest to the negatively charged columns, followed by the 36 kDa isozyme, and then the 27 kDa isozyme. Furthermore, the various isozymes showed up on both sides of the column. For example, The SP bound material contained 27 kDa isozyme as well as the SP unbound material contained 27 kDa isozyme. The columns were only loaded to 70% capacity so overload is not the cause of this. The apparent low PME binding ability to the ion-exchange columns suggest the formation of a PME-pectin complex as the bond between PME and pectin would serve as a competitor to the column(Chen, Sims et al. 1998). The formation of such a complex would "mask" the individual surface charge character of the PME and pectin resulting in poor binding(Chen, Sims et al. 1998). The PME binds to blocks of carboxylic acid groups on the pectin(Nari, Noat et al. 1991). Macdonald, Evans et al. (1993) saw a large spread of pectin enzyme that was alluded with an IEX column due to various molecular weight pectins attaching to the PME. They also observed a dark color (similar in description to the color saw in the PME fractions in this current study) in their PME fractions which they suggested was due to the pectin that was bound to the PME. Previous studies have shown PME-pectin complexes in model systems (Snir, Koehler et al. 1995) and in citrus juice(Hernandez, Chen et al. 1992; Snir, Wicker et al. 1996).Furthermore, formation of PME-pectin complexes explains why our lab has seen different PME extractions based on source of the Valencia orange pulp. For example, an extraction using pulp donated from Citrus World in 2002 showed SPuHPu fraction to contain a combination of 27 and 13 kDa isozyme (Kim, Teng et al. 2005; Kim and Wicker 2006; Kim and Wicker 2007; Hyoungill, Rivner et al. 2008) while this study's Citrus World 2005 pulp SPuHPu contains just the 27 kDa isozyme. The various sizes and molecular weights of the pectins complexed with the PME could be responsible for the differences in elution profiles.

The Valencia PME fractions that were obtained from the column separations are further characterized in **Table 3.1**.

## 3.2 Salt Dependency

The salt dependency test showed the 36 and 27 kDa isozymes were both salt independent as no significant change in activity was noted in the absence of 0.1 M NaCl salt. However, the 13 kDa isozyme appears to be salt dependent as it experienced a ~ 50% decrease in activity with the removal of the salt (**Table 3.2**). These results are interesting when taken with the studies performed by Savary and Cameron's lab groups who used 1.2% (w/v) of NaCl. Their PME peak 2 contained mainly the 36 kDa isozyme with the 13 kDa isozyme and was salt independent(Savary, Hotchkiss et al. 2002). The PME peak 4 contained 36 kDa isozyme and was salt dependent. The results of the 36 kDa peptide in this study appear to match the 36 kDa isozyme peak 2 PME. For optimum activity, 0.25 M salt is needed at pH 8.0 and 0.15 M salt at pH 7.5.

#### 3.3 Heat Stability

The 27 and 13 kDa isozymes retained no activity after heating 10 minutes at 70 °C while the 36 kDa isozyme retained activity Arias and Burns (2002) found a heat stable citrus PME isozyme (which our 36 kDa isozyme matches part of its amino acid sequence- see the following section) that retained its activity after heating 25 mL of PME for 30 minutes at 75 °C. However, Savary, Hotchkiss et al. (2002) heated a 36 and 13 kDa combination of isozymes in a microfuge tube for 5 minutes at 65 °C that did not retain any activity.

## 3.4 Proteomics

Amino acid sequence of the 36 kDa peptide in this study had high homology (Figure3.3). The isozyme both times matched up with a heat stable citrus PME isozyme that was

previously reported by Arias and Burns(2002). Both 36 kDa extractions had high protein scores (186 and 213 respectively). Protein scores for this amino acid sequence greater than 67 are significant (p<0.05). Both extractions showed 19% coverage of the full sequence. It is interesting to note that the matched peptides in the sequence appear to be towards C terminus of the amino acid sequence from the Arias and Burns (2002) sequence and that there were no matched in either run after amino acid 540. Also, interesting to note is that the original sequence from Arias and Burns (2002) has an apparent weight of 70 kDa which is far greater than the mass of the protein of interest. The molecular mass was of the section of the sequence covered was 34,870.487 Da and a pI of 8.7 (as calculated on the website <a href="http://prowl.rockefeller.edu/prowl-cgi/sequence.exe">http://prowl.rockefeller.edu/prowl-cgi/sequence.exe</a>). These results suggest that our isozyme of interest is a cleaved protein from the full 70 kDa sequence which is made up of the C-terminus section of the original protein. However, the sequence determined for 34 kDa isozyme by Savary, Hotchkiss et al. (2002) did not match our sequence at all.

The 27 kDa isozyme was also run on two separate ion exchange column extractions and did not match anything in the NCBI database. The use of a database assumes the protein or peptide being examined has previously been reported, however, the large number of potential sequences make this a very rare case(Bruni 2008). Alternative methods can be applied to identify unknown protein sequence fragments without using a genetics approach.

One such alternative approach is to use the "Expressed Sequence Tags" (EST) NCBI database. The EST database contains sequence data and other information on "single-pass" cDNA sequences from a number of organisms. Our MS/MS spectra were run through this database and returned a match with a protein score of 55 (a protein score of greater than 60 for this amino acid sequence are considered significant at p<0.05). While our protein score of 55 is

less than 60, it is still considered a fairly significant match. The sequence that it matched with is shown in **Figure 3.4**. The spectra had 21% sequence coverage of this protein. However, the coverage fell in the middle section of the amino acid sequence from amino acid 73 to 165 (the full sequence contained 283 amino acids). The protein that matched was from the Washington Navel Orange Thrip-Challenged Flavedo cDNA(Close, Roose et al. 2004). The study was conducted at the University of California, Riverside where they studied young fruits that were placed in a cage with thrips (*Scirtothrips citri*). The fruits were studied after being infected by the thrips and the fruit tissue from the flavedo was sequenced.

Another alternative approach is *de novo* sequencing which returned the same partial amino acid sequence of 14 amino acids on both ion exchange column extractions. The partial *de novo* amino acid sequence returned was VEQL(PG or GP)LNTLGLSA (**Figure 3.5**). This amino acid sequence was not found in any of the sequence returned from the 36 kDa protein. This result is in contrast to the study that said the 34 kDa protein was analogous to the 27 kDa isoform(Savary, Hotchkiss et al. 2002). This partial amino acid sequence was contained in the matching section of the *Scirtothrips* citri match shown in **Figure 3.4**. The partial sequence can be used to create degenerate oligonucleotide for reverse genetic approaches to obtain the full sequence.

The above amino acid sequence was used to conduct a search on MS BLAST to see if the sequence is found in any known sequenced proteins. The sequence was found in several proteins that were mainly used in plant protection. One match was for a protein found in grape that helps fight against fungal infection by regulating the size of the cell wall(Ficke, Gadoury et al. 2004). Another match was for an active enzyme found in tobacco plants that aids in the formation of nectar(Carter and Thornburg 2000).

The 13 kDa isozyme returned a partial de novo amino acid sequence of NQ(VP or

PV)QGSF\_LQER as shown in **Figure 3.6**. There was a gap of about 161 m/z in the spectrum in the middle of the sequence between GSF and LQE. The sequence did not match anything within the database suggesting that this is a protein that has not previously been sequenced. Additionally, this sequence was not found within the sequence from Arias and Burns (2002). All three isozymes were also searched against the amino acid sequence for PME inhibitor(Camardella, Carratore et al. 2000) and returned no matches.

Partial amino acid sequences can be used to develop degenerate oligonucleotides for reverse genetic methods to obtain the full protein sequence. Knowing the full amino acid sequence is important because if one of the isozymes has characteristics that are desirable then the crude enzyme, the isozyme can be recreated in larger quantity through genetic engineering. Furthermore, knowing the sequences can help explain the differing salt dependency and heat stability properties of the differing isoforms.

## 3.3 NMR on modified pectin

<sup>1</sup>H NMR spectra of the modified pectins which vary with dyads and triads in partly esterified galacturonic acid are shown in **Table 3.4**. The control pectin made with 3% solution can be compared to all 3 extractions because the molecular weights of the three extractions were not significantly different (ranging from 135,000 to 240,000 g/mol) (Rivner and Wicker 2008). The spectra featured three main signal groups, the protons H-1, H-4, and H-5 in the G and E residues of ester galacturonans.

There are three isozymes that have been identified with PME activity in citrus fruit in our lab at 36, 27, and 13 kDa(Kim, Teng et al. 2005). Kim and Wicker (2006) found that the PME

fraction that contained 36 kDa and 27 kDa peptides to leave more blocks of continuous charge than the fraction that contained the 36 kDa and 13 kDa peptides (Kim and Wicker 2006).

The data in Figure 3.7 shows the GGG/GGE triad and GG dyad frequencies for all modified pectins and the control pectin. For extraction 1, there were no distinctions between the various pectins modified with individual isozymes. There were also no significant differences between pectin modified with combinations of isozymes. However, the pectins modified with combinations of isozymes had greater GGG/GGE and GG frequencies then pectins modified by individual isozymes. For extraction 2, the pectin modified by the 36 kDa isozyme had a greater frequency of GG than pectin modified by 27 kDa or 13 kDa isozymes whose values are roughly equal. The pectin modified by a combination of 36/27 kDa had a greater GG frequency than the pectin modified with 36/13 kDa isozyme combinations. Pectins modified by isozyme combinations resulted in greater GG frequencies than pectins modified by individual isozymes. For GGG/GGE frequencies, the pectin modified by 36 kDa had a greater frequency of GGG/GGE than pectin modified by 13 kDa isozyme which had a greater frequency of GGG/GGE than pectin modified by 27 kDa. Similar to the GG frequencies, the pectin modified by the 36/27 kDa isozyme combination had a greater frequency of GGG/GGE then the pectin modified by the 36/27 combination of isozyme. For extraction 3, 36 kDa isozyme had a greater frequency of GGG/GGE and GG than 27 kDa isozyme which had a greater frequency of GGG/GGE and GG than 13 kDa isozyme. Throughout the various extractions, there appeared to be a synergistic effect between combinations of isozymes as pectins modified with combinations of isozymes rather than individual isozymes resulted in larger blocks of continuous charge. The control pectin contained the lowest frequency of de-esterified groups in both the triad and the dyad as expected.

Extracting the differing effect the various isozymes had frequency of GGG/GGE and GG is difficult and is further complicated by the pectins having differing %DE. The data in **Figure 3.8** resolves this issue by showing the correlation of frequency of NMR signals of GGG/GGE and GG with % DE of all the pectin samples. %DE accounts for 69.83% of the variation in GGG/GGE frequency while %DE explains 51.78% of the variation in GGG/GGE and GG have a visible linear relationships with % DE and %DE accounts for at least some of the variations in the modified pectin samples.

The data in Figure 3.9 shows the relative frequencies of the triad EEE and EE. For extraction 1, the pectin modified with 27 kDa isozyme had a greater frequency of EEE and EE than the pectin modified with 36 kDa isozyme. The pectin modified with 27/13 kDa isozyme combination had a greater frequency of EEE and EE than the pectin modified with the 26/27 kDa isozyme. Pectins modified by combinations of isozymes had larger frequencies of EEE and EE than pectins modified by individual isozymes. For extraction 2, the pectin modified with 13 kDa isozyme had a greater frequency of EEE than the pectin modified with 27 and 36 kDa isozyme whose EEE frequencies that were not significantly different. The pectin modified with 36/13 kDa isozyme combination had a greater frequency of EEE than pectin modified with 36/27 isozyme combination. For EE frequencies, the pectin modified with 13 kDa pectin was not significantly different than the pectin modified with 36 kDa isozyme. Both pectins had higher EE frequencies than pectin modified with 27 kDa isozyme. The pectin modified with 36/13 isozyme combination had the same frequency as the pectin modified by the 36/27 isozyme combination. For both EEE and EE frequencies, pectin modified with combinations of isozymes resulted in larger frequencies than pectin modified with individual isozymes. For extraction 3, the pectin modified with 13 kDa isozyme had a greater EEE and EE frequency than pectin

modified by 27 kDa isozyme which had a greater EEE and EE frequency than pectin modified by 36 kDa isozyme. The control pectin has more esterified groups than any of the modified pectin.

As before, extracting the differing effect the various isozymes had on frequency of EEE and EE is difficult and is further complicated by the pectins having differing %DE. The data in Figure 10 resolves this issue by showing the correlation of frequency of NMR signals of EEE and EE with % DE of all the pectin samples. %DE accounts for 8.76% of the variation in EEE frequency while %DE explains 86.51% of the variation in EE frequency. EEE and %DE have no correlation at all and does not appear to be effected by %DE has much as the other groups in the NMR spectrum.

NMR is a valuable method for determining action pattern because unlike other methods it is quantitative not qualitative (Grasdalen, Andersen et al. 1996). A recent study utilized Nuclear Overhauser effect spectroscopy NMR to predict degree of random de-esterification and degree of block de-esterification. The study showed NMR was a good tool for this prediction, though the researchers had greater success with random de-esterification than block-wise(Winning, Viereck et al. 2007).

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Figure 3.1. PME isozyme extraction process and fraction characteristics for all extractions. The 13 kDa isozyme binds the strongest to the negatively charged columns followed by the 27 kDa isozyme, and then the 36 kDa isozyme. The same isozyme shows up on both the bound and unbound side of the column because there appear to be pectin-PME complexes forming which change the affinity of the protein for the column. N/m stands for not measurable.



Figure 3.2. Representative NMR spectrum. SPuHPb 9 sample shown is from extraction 2. The peak frequency of peak E and peak G are roughly equal explaining the sample's %DE of 47. The peak GG features a sharp increase as does the GGG/GGE frequency peak from the control pectin. The triads GGG and GGE and the triads EEG and EGE could not be separated due to resolution and are reported together.

Table 3.1. Protein content and enzyme activity of Valencia PME fractions. The bold samples were used to modify pectin sample. The capital letters in the sample ID stand for the abbreviation of the column used and the lower case letters stand for bound (b) and unbound (u). An aliquot of 0.5  $\mu$ g were loaded on the gel prior to silver staining.

Experiment	Sample ID	PME U/ml	pro ug/ul	U/mg	Bands Saw on Gel
	Crude	23.0	0.33	69.4	36,27,13
	Spu	21.2	0.28	74.9	36,27
	Нри	1.2	0.10	12.5	27
	SPb 13	24.2	0.74	32.8	27,13
1	SPbHPb7- 8	99.4	0.55	182.4	27,13
	HPb1-3	4.8	0.20	24.1	27
	SPuHPb7	7.3	0.20	36.7	27
	SPuHPb8	114.5	0.76	151.3	36,27
	SPuHPb9	191.5	0.81	237.0	36
	Crude	29.1	0.47	61.5	36,27,13
	SPuHPb7	10.9	0.11	98.3	27
	SPuHPb8	87.3	0.47	187.7	36,27
2	SPuHPb9	152.7	0.70	219.1	36
	SPuHPb10	87.3	0.45	195.7	36,13
	SPbQu	37.6	0.31	119.7	13
	SPuHPu	3.6	0.08	43.3	27
	Crude	12.1	0.22	55.0	36,27,13
3	SPuHPb8	24.2	0.23	105.0	36
Э	SPuHPu	3.0	0.12	24.5	27
	SPbQu	6.1	0.14	42.5	13

Table 3.2. Enzyme activity of the enzyme sample in the presence and absence of salt The values in () are standard deviations.

lsozyme (kDa)	Enzyme in t of Activity (PME U/mI)	the presence salt U/mg	Enzyme in the Activity (PEU/mI)	Enzyme in the absence of salt Activity PEU/ml) U/mq		
36	17.0 (3.4)	63.3 (12.8)	17.0 (0.0)	63.3 (0.0)		
27	6.7 (0.3)	47.6 (2.0)	6.5 (0.0)	46.2 (0.0)		
13	12.9 (0.0)	59.6 (0.0)	7.3 (0.0)	33.5 (6.3)		

Table 3.3. Monad, dyad, and triad frequencies of Valencia PME modified pectins and pectic fractions. Standard deviations are given in ().

			Dyad (H-1)			Triad (H-5)			Monad (H-4)		
PME used on pectin	kDa of en zyme	Avg of 4 runs % DE	GG	GE	EG	EE	EEE	GGG/GGE	EGG/EGE	E	G
Control Pectin	N/A	60 (5)	0.41 (0.10)	0.24 (0.01)	0.23 (0.00)	0.44 (0.01)	0.54 (0.12)	0.09 (0.01)	0.26 (0.04)	0.60 (0.05)	0.40 (0.04)
EX1 HPb9	36	43 (1)	0.59 (0.03)	0.24 (0.02)	0.20 (0.01)	0.22 (0.02)	0.26 (0.03)	0.29 (0.02)	0.29 (0.01)	0.43 (0.02)	0.57 (0.02)
EX2SPuHPb 9	36	47 (2)	0.58 (0.06)	0.26 (0.02)	0.20 (0.01)	0.32 (0.02)	0.43 (0.07)	0.27 (0.02)	(0.31) 0.02	0.47 (0.02)	0.53 (0.02)
EX3 SPuHPb8	36	32 (3)	0.72 (0.05)	0.19 (0.03)	0.04 (0.01)	0.13 (0.02)	0.48 (0.05)	0.59 (0.04)	0.42 (0.05)	0.32 (0.03)	0.68 (0.07)
EX1 SPuHPb1-3	27	38 (4)	0.52 (0.45)	0.21 (0.18)	0.15 (0.11)	0.17 (0.14)	0.25 (0.24)	0.27 (0.23)	0.25 (0.16)	0.39 (0.34)	0.61 (0.46)
EX1 HPb7	27	47 (6)	0.60 (0.08)	0.27 (0.06)	0.23 (0.02)	0.28 (0.02)	0.41 (0.08)	0.29 (0.01)	0.27 (0.02)	0.47 (0.06)	0.53 (0.07)
EX2 SPuHPb 7	27	50 (5)	0.53 (0.05)	0.32 (0.03)	0.17 (0.02)	0.26 (0.07)	0.40 (0.02)	0.21 (0.03)	0.27 (0.03)	0.50 (0.05)	0.50 (0.03)
EX2 SPuHPu	27	48 (6)	0.46 (0.05)	0.23 (0.02)	0.19 (0.02)	0.28 (0.01)	0.41 (0.06)	0.18 (0.02)	0.32 (0.03)	0.48 (0.06)	0.52 (0.07)
EX3 SPuHPu	27	36 (1)	0.54 (0.02)	0.19 (0.01)	0.16 (0.000)	0.22 (0.00)	0.50 (0.03)	0.28 (0.02)	0.41 (0.03)	0.36 (0.01)	0.64 (0.02)
EX2 SPbQu	13	47 (5)	0.53 (0.03)	0.30 (0.03)	0.23 (0.02)	0.32 (0.02)	0.46 (0.06)	0.26 (0.05)	0.31 (0.05)	0.47 (0.05)	0.53 (0.07)
EX3 SPbQu	13	42 (4)	0.52 (0.03)	0.18 (0.02)	0.16 (0.02)	0.24 (0.02)	0.53 (0.06)	0.28 (0.03)	0.43 (0.02)	0.42 (0.04)	0.58 (0.04)
EX3 Crude Enzyme	36,27,13	35 (4)	0.65 (0.07)	0.22 (0.03)	0.13 (0.02)	0.16 (0.03)	0.42 (0.06)	0.38 (0.01)	0.45 (0.06)	0.35 (0.04)	0.65 (0.07)
EX1 HPb8	36,27	38 (2)	0.64 (0.02)	0.25 (0.03)	0.18 (0.00)	0.20 (0.02)	0.28 (0.03)	0.38 (0.05)	0.29 (0.06)	0.38 (0.05)	0.62 (0.08)
EX2 SPuHPb 8	36,27	49 (4)	0.58 (0.02)	0.27 (0.03)	0.21 (0.01)	0.34 (0.01)	0.43 (0.02)	0.27 (0.03)	0.29 (0.04)	0.49 (0.04)	0.51 (0.03)
EX2 SPuHPb 10	36,13	48 (1)	0.56 (0.04)	0.27 (0.02)	0.22 (0.02)	0.34 (0.04)	0.45 (0.03)	0.23 (0.02)	0.33 (0.02)	0.48 (0.01)	0.52 (0.04)
EX1 SPbHPb7-8	27,13	40 (2)	0.61 (0.02)	0.23 (0.03)	0.17 (0.02)	0.25 (0.01)	0.38 (0.05)	0.35 (0.03)	0.30 (0.05)	0.41 (0.01)	0.59 (0.03)
EX1 SPb13	27,13	41 (3)	0.64 (0.02)	0.25 (0.01)	0.17 (0.01)	0.23 (0.01)	0.27 (0.02)	0.32 (0.02)	0.27 (0.03)	0.40 (0.03)	0.60 (0.02)

	N-terminus				
1	MPYARYLSTL	LHKGPTATAS	QSYEFKRLGQ	SSSYSIYMLH	PCFNLITRNQ
51	ALSTKIFTTM	AQPRKLFLAL	FAVVLVVTAV	VTIIATRKNN	SSNTNDEHIA
101	AHAHAYAQPV	IRSSCSATLY	PELCFSALSA	APAAAVSKVN	SPKDVIRLSL
151	NLTITAVQHN	YFAIKKLITT	RKSTLTKREK	TSLHDCLEMV	DETLDELYKT
201	EHELQGYPAA	ANNKSIAEQA	DELK <mark>ILVSAA</mark>	MTNQETCLDG	<u>FSHER</u> ADKKI
251	REELMEGQMH	VFHMCSNALA	<mark>MIKNMTDGDI</mark>	<mark>GKDIVDHYSK</mark>	ARR <u>LDDETKW</u>
301	<u>PEWLSAGDR</u> R	LLQATTVVPD	<mark>VTVAADGSGN</mark>	<mark>YLTVAAAVAA</mark>	APEGSSRRYI
351	IRIKAGEYRE	NVEVPKK <u>KIN</u>	LMFIGDGRTT	TIITGSR <u>NVV</u>	<b>DGSTTFNSAT</b>
401	VAVVGDGFLA	<u>R</u> DITFQNTAG	<b>PSK</b> HQAVALR	<b>VGSDLSAFYR</b>	<b>CDMLAYQDT</b> L
451	<b>YVHSLR</b> QFYT	<b>SCIIAGTVDF</b>	<mark>IFGNAAAVFQ</mark>	NCDIHAR <b>RPN</b>	PNQRNMVTAQ
501	GR <b>DDPNQNTG</b>	<mark>IVIQK</mark> CR <u>IGA</u>	TSDLLAVKGS	FETYLGRPWK	<u>R</u> YSRTVVMQS
551	DISDVINPAG	WYEWSGNFAL	DTLFYAEYQN	TGAGADTSNR	<b>VKWSTFKVIT</b>
601	SAAEAQTYTA	ANFIAGSTWL	GSTGFPFSLG	L	
					C-terminus

Figure 3.3. 36 kDa isozyme matched against the putative thermostable pectinersterase [*Citrus sinensis*] found by Arias and Burns (2002). The bold matching peptides in extraction 1 and the underlined are the matchining peptides in extraction 2. Both runs had sequence coverage of 19%. The highlighted section of the amino acid sequence has a calculated molar mass of 34,870.487 Da and a pI of 8.7.

	N-terminus				
1	VIQFLIGFAL	WALASSLASA	TDPGHLQDVC	VAINDPKDGV	FVNGKFCKDP
51	KLARAEDFFF	SGLGKPGNTA	NRLGVDETDA	NVEQIPGLNT	LGISAFRIDY
101	APYGQRPPHI	<b>HPR</b> ASEIFLV	LEGTLYVGFV	TSDQLNNTLI	AKVLNK <b>GDVF</b>
151	VFPQGLIHFQ	<b>FNIGK</b> TNAAA	YSALNSQFPG	EVTIADTVFG	ANPSINPDFL
201	GKAFQLDPKI	VKDLQNKFIN	GN_EKELMSF	NINGLFTSGC	ITLSPDYHIL
251	INVIKEVSHE	PLSRLTVAM	_T_LLLFLFS	KKK	
					C-terminus

Figure 3.4. 27 kDa isozyme matched against the Washington Navel Orange Thrip-Challenged

Flavedo cDNA(Close, Roose et al. 2004). The bold red amino acids are the matching peptides.

The run had a sequence coverage of 21%.



Figure 3.5. MS spectra with *de novo* sequence for 27 kDa isozyme fraction.



Figure 3.6. MS spectra with *de novo* sequence for 13 kDa isozyme fraction.



The samples are labeled with their %DE in parenthesis and the isozyme MW is listed at the top of each bar.



Figure 3.8. Correlation of frequency of NMR signals of GGG/GGE and GG with %DE of all pectin samples. Frequency values and % DE values were measured in quadruplicate. Average values were used in the plot. The linear correlation of GGG/GGE frequency with %DE has an  $R^2$  of 0.6983 and the linear correlation of GG frequency with %DE has an  $R^2$  of 0.5178.



Figure 3.9. EEE triad frequencies and EE dyad frequencies of modified pectin samples. The samples are labeled with their %DE in parenthesis and the isozyme MW is listed at the top of each bar.



Figure 3.10. Correlation of frequency of NMR signals of EEE and EE with %DE of all pectin samples. Frequency values and % DE values were measured in quadruplicate. Average values were used in the plot. The linear correlation of EEE frequency with %DE has an  $R^2$  of 0.0876 and the linear correlation of EE frequency with %DE has an  $R^2$  of 0.8651.
## **CHAPTER 4**

# CHARACTERISTICS OF SEPARATED PME ISOZYME MODIFIED PECTIN THROUGH ACTION PATTERN ANALYSIS, CALCIUM SENSITIVITY, AND $\zeta$ -POTENTIAL

## Abstract

Pectin methylesterase (PME) isozymes separated on ion exchange chromatography and isozyme combinations were used to modify pectin. The resulting pectin was evaluated based on molecular weight (Mw), NMR monad, dyad, and triad frequencies, calcium sensitivity, and  $\zeta$ potential. There was no difference observed in Mw between modified and control pectins. In all modified pectin samples, GGG/GGE and GG peaks increased in frequency and the EE peaks decreased in frequency compared to the control pectin. The calcium sensitivity test found a linear correlation between G' and %DE of  $R^2 = 0.568$ . There was a large power correlation observed between G' and GGG/GGE frequencies with an  $R^2 = 0.776$ . There is a much smaller power correlation observed between G' and GG frequencies ( $R^2 = 0.421$ ). A linear correlation was observed between GGG/GGE frequencies and  $\zeta$ -potential (R<sup>2</sup>=0.693). The tests show that both %DE and the distribution of DE play an important role in determining the overall functionality of the pectins. The tests also show that larger blocks of continuous charge affect the functionality of the pectin more than small blocks of charge. However, tests in model systems are needed to see if the minuet observed between modified pectin samples translates into real world differences.

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## 1. Introduction

Pectins are ionically charged structural plant polysaccharides found in the cell wall of many plants and are commonly used in the food industry as gelling, thickening, and stabilizing agents (May 1990; Voragen, Pilnik et al. 1995; Savary, Hotchkiss et al. 2003). Pectin is made up of three major pectic polysaccharides that all contain galacturonic acid (GalA): 1) homogalacturonan (HG), a linear polymer high in GalA content; 2) rhamnogalacturonan I (RGI), a repeating disaccharide with a mixture of glycan chains; 3) rhamnogalacturonan II (RGI) which features GalA resides and numerous structurally different oligosaccharides side chains all linked to the RGII backbone; (Ridley, O'Neill et al. 2001). The side chains contain primarily Larbinose, D-galactose, and D-xylose (De Vries, Rombouts et al. 1982; De Vries, den Uijl et al. 1983). The GalA residues that make up the pectin chain can be O-acetylates at HO-2 and/or HO-3 or methyl esterified at C-6(Nelson, Smit et al. 1977). The degree of esterification (% DE) at this position classifies pectin between low-methoxyl (25-50% DE) and high-methoxyl (50-80% DE). Vinvken, Schols et al. (2003) proposed another view of the pectin structure in which HG would be a side-chain of RG-I.

The industrial application of pectin is largely determined by the methyl-esterification of HG. The properties and biological functions of HGs are thought to be determined by ionic interactions(Ridley, O'Neill et al. 2001; Willats, McCartney et al. 2001). Daas, Meyer-Handsem, Schols, De Ruiter, & Voragen (1999) first presented the term 'degree of blockiness' as the total amount of liberated GalA residues expressed as the proportion of the total number of free GalA residues percent in the pectin. Both the %DE and the distribution of the methyl groups or degree of blockiness have a large influence on the properties of pectin. For example, a block-wise arrangement of free carboxyl groups leads to improved calcium-gelling properties when

compared to pectins with a random distribution of free carboxyl groups(Thibault and Rinaudo 1985; Ralet, Dronnet et al. 2001).

Pectin gelation occurs in LMP when calcium bridges form between two carboxyl groups in two differing chains in close contact(Axelos and Thibault 1991; Voragen, Pilnik et al. 1995). The pattern of esterification, block-wise or random, impacts the aggregation and association properties of the pectin towards cations such as calcium (Powell, Morris et al. 1982; Willats, McCartney et al. 2001). Modifying the charge and charge density of a pectin effects the gelling properties(De Vries, Hansen et al. 1986; Willats, Orfila et al. 2001) and calcium binding capability(Limberg, Korner et al. 2000). Pectin gelation properties are impacted by the length of their side branches and degree of acetylation(Matthew, Howson et al. 1990). Additionally, the charge of the pectin also affects the ability of the pectin to act as a stabilizer in low pH environments (Glahn 1982).

The enzyme that catalyses the demethoxylation of esterified pectin is pectin methylesterases (PME, E.C..3.1.1.11). Various studies have revealed that plant PME's demethylate pectin leaving blocks of de-esterified groups (charges) along the pectin backbone(Limberg, Korner et al. 2000; Daas, Voragen et al. 2001; Hotchkiss, Savary et al. 2002; Savary, Hotchkiss et al. 2002; Kim, Teng et al. 2005; Kim and Wicker 2006). In Valencia orange PME, different individual isozymes in the PME can be characterized by expression patterns, physical, and biochemical properties (Bordenave 1996; Savary, Hotchkiss et al. 2002).

There have been several methods used to reveal the variations in action patterns of differing PME. The blocks of charge on pectins have been analyzed by enzymatic methods employing exo-polygalacturonase (exo-PG ) and endo-polygalacturonase (EPG) (Limberg, Körner et al. 2000). Pectin lyase, which cleaves the HG in high methylated regions, was used to

estimate lengths of partially esterfieid blocks by Limberg, Korner, et al. (2000). Distinction between pectins modified with plant PME (block-wise demethylation) and fungal PME or base catalysis (random demethylation) in respect to the action pattern of the demethylation have been shown with enzymatic fingerprinting with EPG(Daas, Voragen et al. 2001) and EPG II, as well as methods using specific pectin antibodies(Willats, Limberg et al. 2000). EPG has also been used to study action patterns by looking for blocks of charge on the pectins(Daas, Meyer-Hansen et al. 1999). Capillary zone electrophoresis was utilized to analyze %DE and found there was a correlation between % DE and migration times of pectin (the higher the %DE, the shorter the migration time(Jiang, Liu et al. 2005). Zhong, Williams, Keenan, Goodall & Rolin (1997) also found a linear relationship between %DE and migration time and in a second study Zhong, Williams, Goodall, and Hansen(1998) developed a method to interpret capillary electrophoresis peaks to determine distribution of %DE.

Nuclear magnetic resonance (NMR) studies have revealed variations in action patterns of these differing PME isozymes combinations (Grasdalen, Andersen et al. 1996; Kim, Teng et al. 2005). Indirect statistical methods on GalA oligomers with differing %DE's measured with NMR have been used to measure the blocks of charge on pectin(Catoire, Pierron et al. 1998; Denes, Baron et al. 2000). Colquhoun, de Ruiter, Schols and Voragen (1990) used 2D- NMR to analyze the oligosaccharides on the hairy region of modified apple pectin. A recent study utilized Nuclear Overhauser effect spectroscopy NMR to predict degree of random deesterification and degree of block de-esterification. The study showed NMR was a good tool for this prediction, though the researchers had greater success with random de-esterification than block-wise(Winning, Viereck et al. 2007). NMR is a good method for determining action

pattern because unlike other methods it is quantitative not qualitative and non destructive to the sample (Grasdalen, Andersen et al. 1996).

The current study concentrates on examining the functional properties of modified pectin. The researchers hypothesis that differing PME isozymes will give different charges and charge distributions that will influence the functionality of pectin. The goal of this paper is to associate the structural properties of the pectin (such as %DE, blocks of charge, overall charge on the shear plane of the particle, and calcium sensitivity) with the pectin's functional properties (defined as G' in this study). Knowing how the structure of the pectin affects the pectin's functional properties is important to build designer pectin that has specific qualities. Knowing which individual PME isozyme or isozyme combination results in these desirable qualities is important because this will allow researchers to pick the isozyme that gives them their desirable pectin qualities.

## 2. Materials and Methods

## 2.1 Materials

Crude Valencia PME was extracted from Valencia orange pulp from 2005 processing season (donated by Citrus World, Lake Wales, Fl). The pectin used was commercial high methoxyl pectin (GENU pectin type B rapid set-z, Batch G 32 691, CP Kelco, Lille Skensved, Denmark).

## 2.2 Valencia PME Preparation

The crude PME extract was produced following the method described by Wicker, Vassallo et al. (1998). The crude PME extract was then separated into various fractions following the method described by Rivner, Urbauer, and Wicker (2008). The fraction collected and the columns used in the extractions are identified in **Figure 4.1**. Notice that extraction 1

differed from extraction 2 and 3 by employing a Heparin cation exchange column after the SP column instead of a Q anion exchange column.

#### 2.3 Pectin modification

The 36, 27, and 13 PME isozyme and isozyme combination fractions along with the curde enzyme were used on citrus pectin to de-esterify the pectin to a target %DE. In extraction 1 and 2, the target DE was 10% lower than control pectin. In experiment 3, the target DE was 20% lower than the control. The modified pectins and control pectin were prepared following the method by Rivner, Urbauer, and Wicker (2008).

## 2.4 Characterization of Modified Pectin

## 2.4.1 HPSEC – Multi Angle Light Scattering

Molecular weights (Mw) were measured following the meothod described by Corredig and others (2000) and Kim and others (2005) by means of an HPSEC-multi angle light scattering system consisting of a Waters P515 pump equipped with an in-line degasser (Waters, Milford, MA) and two in-line filters (0.22 and 0.11 nm pore size, Millipore, Bedford, MA). Pectin dispersions in 50 mM sodium nitrate (3 mg/ml) were filtered through 0.8 µm (polypropylene, 25 mm, Whatman, Maidstone, England). 50 mM sodium nitrate mad up the mobile phase and was filtered through 0.2, 0.1, and 0.1 µm filters (47 mm, Gelman Science, Ann Arbor, MI). A guard column and two PL-Aquagel-OH linear mix columns (8 µm pore size, Polymer Laboratories, Inc., Amherst, MA) were connected in series to attain separation. A multi-angle light scattering detector DAWN DSP-F equipped with a P10 flow cell and a HE-NE laser-light source (633 nm) and an Optilab DSP interferometric refractometer operating at 633 nm (Wyatt Technologies, Santa Barbara, CA) were coupled in series. ASTRA/Easi SEC software (version 4.74.03) was used to process Mw as a number average (Mn), weight average (Mw) and z-average (Mz) for each sample. The samples were run in duplicate and averaged together. The specific refractive index increament value used was the same as used by Kim and others(2005).

## 2.4.2 NMR on modified pectin

The %DE and distribution of % DE of the pectin was estimated by <sup>1</sup>H NMR spectroscopy in accordance to Rivner, Urbauer, and Wicker (2008). , The NMR peak assignments used were also from Rivner, Urbauer, and Wicker(2008) and are featured in **Figure 4.3**. An example of the frequency table generated from the NMR spectra is given in **Figure 4.4**.

#### 2.4.3 Modified Pectin Rheological Measurement

The viscoelastic properties of pectin gel were measured on the modified pectin samples and control pectin using a Rheometric Scientific SR5000 Controlled Stress Dynamic Rheometer<sup>TM</sup> (Rheometrics, Piscataway, New Jersey, U.S.A.) outfitted with a cone and plate apparatus (60 mm diameter,  $0.0385^\circ$ , 0.5 mm gap). The experiment was conducted at  $20.2^\circ$ C. The measurement was taken by inducing gelation directly on the rheometer plate by mixing 1.2ml of 2% pectin sample with stock 90.3 µl of 500 mM CaCl<sub>2</sub> and pre-shearing for 30s at 5 Pa. The gel was allowed to equilibrate for 10 minutes before the storage modulus G' and the loss modulus G'' were measured using a dynamic frequency sweep test at a controlled stress of 5 Pa with an initial frequency of 0.1 Hz and a final frequency of 10 Hz. This test was repeated twice for each sample. The 5 Pa controlled stress that was applied was verified to be in the linear regime.

## 2.4.4 $\zeta$ -potential of modified pectin

The charge on the shear plane of the particle was analyzed by measuring the  $\zeta$ -potential. This measurement was accomplished by means of a Particle Size Analyzer adding the BI-Zeta option (90 Plus, Brookhaven Inst., Holtsville, NY) with a 50 mV diode laser (90° angle) and a

BI-9000AT correlator(Nakamura, Furuta et al. 2003; Kim and Wicker 2007; Hyoungill, Rivner et al. 2008). Both 0.1% and 0.05% (w/w) pectin dispersions were made in 0.01 M NaPO<sub>4</sub> buffer at pH 7.0 for all 3 experiments.

The particle size of the pectin solution was determined using the 90 plus instrument. The effective diameter of the particles in the pectin solutions was calculated from a collective fit of the intensity autocorrelation function acquired from the intensity fluctuation of the scattered light with Brookhaven Instrument Particle Sizing Software (version 3.37, Brookhaven Instruments, Worcestershire, UK). Each sample was run five times. The particle size was then used to calculate the  $\zeta$ -potential with Brookhaven Instrument Zeta Potential Analyzer Software (version 3.23, Brookhaven Instruments, Worcestershire, UK). The  $\zeta$ -potential runs were ran at 25°C with the laser beam set to 659.0 nm and 1.330 refractive index. Each concentration was run five times. The results from the two concentrations were averaged together and reported as a mean value for  $\zeta$ -potential.

#### 2.5 Statistical Analysis

All statistical analysis was run in Minitab 15. Microsoft Excel 2007 was used to organize the data in spreadsheets and for simple regression analysis.

#### **3 Results and Discussion**

## 3.1 PME isozyme separation

The PME isozymes were successfully separated. A series of SP, HP, and Q ionic exchange columns were used to achieve this goal (**Figure 4.1**). Extraction 1 varies from extraction 2 and 3 by using a Heparin affinity column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) after the Hi-Trap SP cation exchange column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) instead of a Hi-Trap Q HP anion exchange column (GE Healthcare Bio-

Sciences AB, Uppsala, Sweden). The isozymes were characterized based on their molecular weight band and their units of activity (**Table 4.1**).

## 3.2 Molecular Weight

The Mw of the modified pectin ranged from 135,000 g/mol to 240,000 g/mol (except for Extraction 3 SPbQu which had a Mw of 268,000 g/mol) with the control pectin having a Mw of 196,000. This is in accordance to the results of Kim and others (2005) who had Valencia PME charge modified pectin ranging from 130,000 g/mol to 211,000 g/mol. There was also no significant change in Mw between pectins made with 1% solution (extraction one and two) and 3% pectin (extraction three)(**Figure 4.2**). These results are also in agreement with several other studies that showed conservation of Mw after orange PME de-esterification despite which PME isozyme employed (Cameron, Savary et al. 2003; Hunter, Thomas et al. 2006).

## 3.2 Nuclear Magnetic Resonance

<sup>1</sup>H NMR spectra were collected and analyzed by measuring the area under the monads, dyads, and triads of esterified (E) or carboxylic acid (G) groups (**Figure 4.2**). In all the samples, the GG and GGG/GGE peaks increased in size and the EEE peaks decreased in size between the control pectin and the modified pectin samples. An example of all the peak frequencies compared to the control pectin can be found in **Table 4.2**. The isozymes that bound to the Heparin column caused the greatest shift in signal between the control and the resulting modified pectin in terms of the dyad and triad carboxylic acid NMR signals.

The %DE was also calculated from the <sup>1</sup>H NMR spectra and this can be seen in **Table 4.1**. Experiment 1 featured a range of %DE from 47-38, Experiment 2 featured a tighter range of %DE from 50-47%, and Experiment 3 featured a range from 32-36 %DE (with one sample falling out of range at 42%DE).

## *3.3 Calcium Sensitivity*

The samples were evaluated based on their storage and loss moduli, G' and G'' which are valuable parameters for evaluating gel strength. G', the storage modulus, is defined as the ratio of stress in phase with the strain to the strain. G'', the loss modulus, is defined as the ratio of the shear stress 90° out of phase with the strain to the strain(Bourne 2002). In other words, G' is a measurement of the energy stored in the gel while G'' is the apparent viscous section in the gel. Strong gels characteristically feature G' values much larger than their G'' values. The larger the G' value, the stronger the gel strength and the greater the pectin is binding to the calcium to form cross linking.

All of the samples formed gels in the presence of the calcium ion except for the control pectin which featured low G' and G'' that were relatively close in range to each other (G'=40 and G''=6.3). De-esterfication of pectin resulted in Ca<sup>++</sup> sensitive pectin as observed by the increase in G' over the control pectin (**Table 4.1**). According to the linear regression model, 56.8% of the variation in G' can be attributed to the change in %DE (**Figure 4.4**). For %DE between ~47 and 50% DE, the G' value ranged from 1141.85 Pa to 3570.5 Pa with the weakest G' coming from Extraction 2 SPuHPu fraction, followed by SPbQu, and the largest G' coming from the pectin modified with the enzyme that bound to the HP column (SPuHPb). A 10% decrease in %DE from ~47 to ~38% resulted in modified pectin that ranged from 3218 Pa (Extraction 1 SPbHPb7-8) to 4399.55 (Extraction 3 SPbQu). A further decrease from ~38 to ~32 resulted in G' from 2681.5 (Extraction 3 CE) to 5983.35 (Extraction 1 SPuHPb8). It is interesting to note that the points on the graph seem to have more of a "step like" trend to them then a linear regression. There seems to be three groups of points on the graph with the first group centered at 48% DE, the second group at 41% DE, and the third group at 35% DE. When

the graph is looked at from this viewpoint, it appears that as the %DE is lowered, there is a step up in G' average value but that individual points in the groupings may be lower or higher than the previous %DE group.

The Mw of the pectin had no apparent influence on the  $Ca^{++}$  properties of the pectin (**Figure 4.5**). The R<sup>2</sup> value for a linear regression model is 0.008 showing that there is no relationship. It is important to note that the size of the molecules (which were not significantly different) did not have an effect on the magnitude of G'.

According to the power model shown in Figure 6, 77.6% of the variation in G' in all three extractions can be attributed to GGG/GGE peak area frequencies in the NMR spectra (**Figure 4.6**). **Figure 4.7** shows the relationship between G' and GG peak area frequencies (0.421) which is smaller than the relationship between G' and GGG/GGE peak area frequencies ( $R^2$ =0.776). This suggests that the Ca<sup>++</sup> binding properties are greater effected by larger regions of de-esterfied blocks than short ones.

**Figure 4.8** shows the measured G' (Pa) at various combination of %DE and GGG/GGE peak area frequencies. At first, the %DE decreases while the GGG/GGE peak area frequencies increase causing an increase in G'. Then GGGG/GGE peak area frequencies holds steady while %DE decreases from ~40 to ~35 which results in very little change in G' values. Then the GGG/GGE peak area frequencies increases while the %DE holds constant yielding an increase in the G' value. The trend line presented in this graph suggests that the blocks of charge have a greater impact on G' values then %DE because when the GGG/GGE peak area frequencies are constant and %DE changes, the G' values hold constant as opposed to when %DE holds constant and GGG/GGE peak area frequencies increase yielding an increase in G' value.

A Pearson's correlation matrix was created to further evaluate the correlations between G', G'', %DE, GGG/GGE peak area frequencies and GG peak area frequencies (**Table 4.3**). G' and G'' had a high correlation(0.885). GGG/GGE peak area frequencies had a moderate correlation with G' (0.553) and a slightly improved correlation with G'' (0.663). G' had a weak correlation with GG peak area frequencies (0.447), a stronger correlation with G'' (0.503), and a strong correlation with GGG/GGE peak area frequencies (0.892). % DE had a strong negative correlation with G' (-0.745), and slightly stronger negative correlation with G'' (0.762), a stronger negative correlation with GGG/GGE peak area frequencies (-0.735). It is important to keep in mind that these correlations are for linear relationships which may not be the best fit for the data (as evident in Figure 9). Some of the correlations would better fit on an exponential or power fit line.

# 3.4 $\zeta$ -potential of modified pectin

ζ-potential is a measurement of the magnitude of the repulsion or attraction between particles, and this measurement sheds lights on the dispersion mechanism and is crucial for electrostatic dispersion control (Farinato and Dubin 1999). ζ-potential was run on the modified pectin from extractions 2 and 3. The modified pectins from extraction 1 were not tested due to insufficient amount of sample. Analysis of the data showed a strong linear correlation between ζ-potential and NMR dyads and triads of carboxylic acid (G) groups (**Figure 4.10 and Figure 4.11**). The linear regression analysis of extraction 2 GG peak area frequencies versus ζ-potential exhibited an R<sup>2</sup> value of 0.897 (slope= -28.94; intercept= -17.34) and GGG/GGE peak area frequencies versus ζ-potential yielded an R<sup>2</sup> value of 0.949 (slope= -39.81; intercept= -23.35). The linear regression analysis of extraction 3 GG peak area frequencies versus ζ-potential

exhibited an R<sup>2</sup> value of 0.786 (slope= -0.0008; intercept= 0.302) and GGG/GGE peak area frequencies versus  $\zeta$ -potential yielded an R<sup>2</sup> value of 0.770 (slope= -0.011; intercept= -0.074). When the data points from both extractions are combined for linear regression analysis GG peak area frequencies versus  $\zeta$ -potential exhibited an R<sup>2</sup> value of 0.708 (slope= -84.91; intercept= 13.75) and GGG/GGE peak area frequencies versus  $\zeta$ -potential yielded an R<sup>2</sup> value of 0.693 (slope= -56.61; intercept= -17.17).

**Table 4.4** features a Pearson's correlation matrix to further assess the correlations between  $\zeta$ -potential, G', G'', %DE, GGG/GGE peak area frequencies and GG peak area frequencies. Only pectins modified from extractions 2 and 3 were considered for this table as there were insufficient quantities of extraction 1 modified pectin to run  $\zeta$ -potential.  $\zeta$ -potential had strong correlation with %DE (0.795), high negative correlation with G' (-0.700) and strong negative correlations with G'' (-0.728), GGG/GGE peak area frequencies (-0.835), and GG peak area frequencies (-0.833). These correlations are for linear relationships which may not be the best fit for the data (as evident in **Figure 4.12**). Some of the correlations would better fit on an exponential or power fit line.

The  $\zeta$  potential measurement allows us to use the predictive method of emulsion stability(Mengual, Meunier et al. 1999). In other words, the larger the negative charge on the hydrocolloid, the more the charge will impede upon coalescence and increase stability (Morrison and Ross 2002). Pectin treated with enzyme treatments tend to have higher negative  $\zeta$ -potential than non treated pectin due to the galacturonic acids that were digested from the main pectin backbone leaving exposed charges(Kim and Wicker 2007).

This data is important because it represents evidence that  $\zeta$ -potential can be used as a quick screen for GG peak area frequencies and GGG/GGE peak area frequencies in NMR.  $\zeta$ -potential

is a much easier, quicker and cheaper procedure than NMR. These results indicate that  $\zeta$ -potential can be a valuable alternative to NMR for a large sample set of data.

## 4. Conclusions

The tests throughout the study showed that both the %DE and the distribution of DE played an important role in determining the overall functionality of the pectin (as measured by the calcium sensitivity test). The larger the blocks of continuous charge on the pectin backbone, the greater effect the blocks had on the functionality of the pectin. This was evident from the NMR frequency of GGG/GGE having a much greater correlation with G' then the GG frequency. However, since the relationship was best described with a power function (as opposed to a linear trend), it is hard to determine how much an increase in block charges really effect the overall functionality of the pectin. In other words, the actual differences seen in the pectin might be too minuet to see an actual distinction in a real world test. To further evaluate the functionality of each individual isozyme, a future study is needed that tests pectins modified from the specific isozymes used in a model system and evaluated based on their real world functionality.

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Figure 4.1. PME isozyme extraction process and fraction names. Extraction 1 differs from Extraction 2 and 3 by the use of a Heparin column after the SP column instead of a Q column.

Name	Isozy me	Activity (U/mg)	% D E	G' (Pa) at 4 Hz	G" (Pa) at 4 Hz	G G	GG G/ GGE	ζ- potential
СР	none	N/A	60	40	6	0.4 1	0.09	-12
Ex1SPuHPb 9	36	506	43	2002	103	0.5 87	0.29 3	not measured
Ex2SPuHPb 9	36	219	47	2604	133	0.5 81	0.26 7	-34
Ex3SPuHPb 8	36	136	32	3965	462	0.7 2	0.59	-45
Ex1SPuHPb 7	27	72	47	3861	240	0.5 03	0.28 6	not measured
Ex1HPb1-3	27	18	38	5322	539	0.6 5	0.30 6	not measured
Ex2SPuHPb 7	27	98	50	2121	105	0.5 28	0.20 9	-32
Ex2SPuHPu	27	43	48	1142	52	0.4 56	0.27	-31
Ex3SPuHPu	27	37	36	4219	237	0.5 4	0.28	-28
Ex2SPbQu	13	120	47	2105	101	0.5 28	0.27	-33
Ex3SPbQu	13	19	42	4400	200	0.5 2	0.28	-39
Ex3CE	36,27, 13	58	35	2682	212	0.6 5	0.38	-42
Ex1SPuHPb 8	36,27	1157	38	5983	466	0.6 23	0.34 8	not measured
Ex2SPuHPb 8	36,27	188	49	2505	125	0.5 83	0.20 9	-34
Ex2SPuHPb 10	36,13	196	48	1452	67	0.5 59	0.26 7	-30
Ex1SPbHPb 7-8	27,13	201	40	3219	216	0.6 05	0.32 9	not measured
Ex1SPb13	(27),1 3	33	41	3943	245	0.6 41	0.32 3	not measured

Table 4.1. Summary of measurements taken in all 3 extractions



Figure 4.2. Cumulative weight fraction plotted against molecular weight of modified pectins.



Figure 4.3. Representative NMR spectrum showing the control pectin versus pectin modified with extraction 2 SPuHPb9. The triads GGG and GGE as well as the triads EGG and EGE could not be separated due to resolution and are reported together. Notice that the area of G peaks expands and the E peaks area shrink when the pectin is modified.

Table 4.2. Representative NMR frequency table showing control pectin and SPuHPb9 from experiment 2. The triads GGG and GGE as well as the triads EGG and EGE could not be separated due to resolution and are reported together. The numbers reported are the average of 4 sets of 256 NMR scans. The standard deviation is given in parenthesis. The frequencies for groups containing G increase while groups containing E decrease between control pectin and PME modified pectin sample.

PME used on pectin	GE	GG	EEE	EE	EG	GGG/ GGE	EGG/ EGE	E	G	%DE
pecun	0L	UU	LEL		LO	UUL	LUL		U	
Control	0.24	0.41	0.54	0.45	0.23	0.09	0.26	0.6	0.41	60
Pectin	(0.01)	(0.1)	(0.12)	(0.01)	(0)	(0.01)	(0.04)	(0.05)	(0.4)	(5)
Ex2	0.26	0.58	0.43	0.32	0.2	0.27	0.31	0.47	0.53	47
SPuHPb	(0.02)	(0.06)	(0.07)	(0.02)	(0.01)	(0.02)	(0.02)	(0.02)	(0.02)	(2)



Figure 4.4. Linear correlation of G' (Pa) of 2% pectins in the presence of 35 mM calcium at 4 Hz and 5 Pa at 20.2 °C with % DE for all 3 extractions. G' were measured in duplicate and %DE was measured in quadruplicate. Average values were used in the plot. The line that best fits the data features an  $R^2$  value of 0.568. The data appears to be split into three main clusters (circled on the figure) that increase in G' in a step wise fashion.



Figure 4.5. Linear correlation of Mw (g/mol) with the G' (Pa) of 2% pectin in the presence of 35 Mm calcium at 4 Hz and 5 Pa at 20.2  $^{0}$ C for all 3 extractions. Mw and G' values were measured in duplicate. Average values were used in the plot. There is no relationship apparent between these two variables as the R<sup>2</sup> value is 0.008.



Figure 4.6. Correlation of G' (Pa) of 2% pectins in the presence of 35 mM calcium at 4 Hz and 5 Pa at 20.2 °C with frequency of GGG/GGE in NMR spectra in all 3 extractions. G' values were measured in duplicate and GGG/GGE frequency in NMR spectra were measured in quadruplicate. Average values were used in the plot. The data fits the power curve with an  $R^2 = 0.776$ .



Figure 4.7. Correlation of G' (Pa) of 2% pectins in the presence of 35 mM calcium at 4 Hz and 5 Pa at 20.2 °C with frequency of GG in NMR spectra in all 3 extractions. G' values were measured in duplicate and GG frequency in NMR spectra were measured in quadruplicate. Average values were used in the plot. The  $R^2$  is 0.421.



Figure 4.8. Effects of %DE and GGG/GGE on G' (Pa).

0.885			
0 550			
0.553	0.663		
0.447	0.503	0.892	
-0.745	-0.762	-0.831	-0.735
	0.553 0.447 -0.745	0.553         0.663           0.447         0.503           -0.745         -0.762	0.553       0.663         0.447       0.503       0.892         -0.745       -0.762       -0.831

Table 4.3. Correlation Matrix between G' (Pa), G'' (Pa), GGG/GGE, GG, % DE in Extraction 1, 2, and 3.



Figure 4.9. Correlation matrix for G' (Pa), G'' (Pa), GGG/GGE, GG, % DE in extractions 1-3.



Figure 4.10. Correlation of  $\zeta$ -potential with GGG/GGE frequency in NMR spectra for experiment 2 and 3.  $\zeta$ -potential were measured in duplicate and GGG/GGE frequency in NMR spectra were measured in quadruplicate. Extractions 2 and 3 fit on the same linear model that has an R<sup>2</sup> value of 0.689.



Figure 4.11. Correlation of  $\zeta$ -potential with GG frequency in NMR spectra for experiment 2 and 3.  $\zeta$ -potential were measured in duplicate and GG frequency in NMR spectra were measured in quadruplicate. Extractions 2 and 3 fit on the same linear model that has an R<sup>2</sup> value of 0.692.

Table 4.4. Correlation Matrix between G' (Pa), G'' (Pa), GGG/GGE, GG, % DE, and  $\zeta$ -potential in Extraction 1, 2, and 3. Extraction 1 was excluded because  $\zeta$ -potential was not measured due to a limit of sample.

	G'	<b>G</b> ''	GGG/GGE	GG	%DE
<b>G</b> ''	0.795				
GGG/GGE	0.682	0.950			
GG	0.597	0.822	0.930		
% DE	-0.816	-0.872	-0.860	-0.780	
ζ-potential	-0.700	-0.728	-0.835	-0.833	0.795



Figure 4.12. Correlation matrix between all the measured factors in extractions 2 and 3. Extraction 1 was excluded because  $\zeta$ -potential was not measured due to a limit of sample.
# **CHAPTER 5**

# IMPACT OF PECTIN CHARGED MODIFIED BY SEPARATED VALENCIA ORANGE PME ISOYMES AND WHEY PROTEIN COMPLEXES ON OIL-IN-WATER EMULSION STABILITY

# Abstract

Pectins charge modified by separated pectin methelesterase (PME) isozymes were used to form complexes with whey protein to test their ability to stabilize an oil-in-water emulsion. No differences in emulsion activity (EA) measurements were observed between emulsions formulated with modified pectin samples. Initial EA stability was higher in emulsions with pectin and protein versus just pectin or just protein. Back scattering (BS) profiles of the emulsions showed the samples were subject to creaming, phase separation, and coalescence or flocculation.

#### **1.0 Introduction**

To achieve increased stability of a food system, proteins are used in the industry as emulsifying agents and polysaccharides are used as stabilizing agents. A stable emulsion features droplets that are barred from joining together by electrostatic or steric stabilizations. The droplet surface must be fully covered or bridging flocculation will occur (Benichou, Aserin et al. 2002).

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Mixtures of both biopolymers tend to cause a synergistic effect. The covalent proteinpolysaccharide complex enhances stability by forming a thicker, stronger steric-stabilizing layer around the droplets than just a protein alone would(Tokaev, Gurov et al. 1987; Benichou, Aserin et al. 2002). The overall stability of the mixture depends not only on the functional properties of the distinct components, but also on the features and potency of the protein-polysaccharide interactions(Dickinson 1995). The attractive forces between the biopolymers results in soluble complexes. Conversely, if the biopolymers bear similar charges, the complex will be thermodynamically opposing and can lead to segregative phase separation if the polymer concentration is larger than the co-solubility limit(de Kruif and Tuinier 2001).

There is a large interest in replacing synthetic emulsifiers with natural ingredients such as pectin (Garti 1999). Pectins are ionically charged structural plant polysaccharides found in the cell wall of many plants and are commonly used in the food industry as gelling, thickening, and stabilizing agents(May 1990; Savary, Hotchkiss et al. 2003). Pectin methylesterase (PME, E.C..3.1.1.11) catalyses the demethoxylation of esterified pectins. The degree of esterification (% DE) at the C6 position on the pectin chain classifies pectin between low-methoxyl (25-50% DE) and high-methoxyl (50-80% DE). In citrus, multiple isozymes of PME are present. Initially there were three isozymes described in orange with one featuring a high molecular weights and thermal stability(Versteeg, Rombouts et al. 1980). Separating these differing isozymes is important because they most likely have differing action patterns and therefore effect pectin different(Kim, Teng et al. 2005). Distribution of the methyl esters along the complex pectin structure plays a key role in determining the functionalities of the pectin(De Vries, Hansen et al. 1986).

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Pectin is a strong emulsifier because of its ability to ease formation, increase stability, and yield favorable physicochemical properties in oil-in-water (o/w) emulsions (Dickinson 2003; McClements 2004). Additionally, pectin is commonly used to stabilize acidified milk drinks(Tromp, de Kruif et al. 2004). This stabilization is accomplished through pectin reacting with casein and preventing the aggregation of the protein at pH below the isoelectric point (IEP) of casein (pH 4.6). Many common acidified drink products are formulated at pH 4.2 (Tromp, de Kruif et al. 2004). Furthermore, at pH above the pI of the protein, complexes do not form with globular proteins(Tolstogusov 1986). Einhorn-Stoll, Glasenapp, & Kunzek (1996) found that pectin and protein complexes produced greater stabilization at pH 4 than at pH 7. Casein adsorbs to pectin at pH below 5.0 showing that the interaction is primarily electrostatic(Tuinier, Rolin et al. 2002). Another study found that interactions between both low and high methoxyl pectin and  $\beta$ -lactoglobulin to be largely caused by electrostatic interactions(Girard, Turgeon et al. 2002).

Einhorn, Glasenapp & Kunzek(1996) reported that the lower %DE in LMP, the more pectin whey protein complexes could be formed improving emulsion stability. Another study by Lee, Rivner, Urbauer, Garti, & Wicker (2008) showed that modified pectin with %DE between 30-50% showed no difference in calcium sensitivity. The current study tests the pectin-protein complexes of charge modified pectins described in a previous study that were created using the separated isozymes of pectin methylesterase (PME). The study aims to investigate whether pectins with similar %DE but differing distribution of DE stabilize emulsions differently. Emulsion will be made at pH typically found in acidified milk drinks containing both modified pectin and whey protein and tested for their emulsion quality using various methods.

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#### 2. Materials and Methods

## 2.1 Materials

Crude Valencia PME was extracted from Valencia orange pulp from 2005 processing season (donated by Citrus World, Lake Wales, FL). Commercial high methoxyl pectin (GENU pectin type B rapid set-z, Batch G 32 691), was donated by CP Kelco (Lille Skensved, Denmark). Whey protein isolate (IsoChill 9000, Lot # CS43563E) was provided by Trega Foods Inc (Luxemburg, WI).

#### 2.2 Valencia PME Preparation

The crude PME extract was created following the method described by Wicker and others (1998) in 0.1 M NaCl, 0.25 Tris buffer at pH 8.0. The crude PME extract was then separated into different fractions using a series of ionic exchange columns detailed by Rivner and others (2008). The chromatography start buffer was 0.05 M sodium phosphate, pH 7.0 buffer and elution buffer included 1 M NaCl.

## 2.3 Pectin modification

The pectins and control pectins were prepared in accordance to the method in Rivner and others (2008). A calculated amount of 36, 27 and 13 PME isozyme fractions and crude enzyme was used to de-esterify 3% citrus pectin to a targeted 20% DE decrease lower than the unmodified pectin, in approximately 35 minutes. The activity of the isozymes was stopped by decreasing the pH to 4.5, adding 95% ethanol to a final concentration of 80% ethanol, and boiling for 10 minutes. Pectin was created using the crude enzyme that contained 36, 27, and 13 kDa isozymes, SPbHPb9 enzyme that contains 36 kDa isozyme, SPuHPu enzyme that contains 27 kDa isozyme and SPbQu enzyme that contains 13 kDa isozyme. In this paper, the PME

fractions are referred to as the isozymes they contain (i.e., SPuHPu is referred to as 27 kDa isozyme).

## 2.4 Emulsion Preparation

1% whey protein solution was formulated by dissolving whey protein into aqueous solution at pH 2.0 (adjusted with HCl). The pH was held for 10 minutes and then increased to 4.2 (adjusted with 4N NaOH) as described by Larichev, Gurov & Tostoguzov (1983). This pH adjusting procedure allowed the achievement of soluble protein in which was used to create protein-pectin complexes (Larichev, Gurov et al. 1983). A 15 ml aliquot of 1% pectin stock solution in water was made from each pectin of interest. The pectin solution and the whey protein solutions were stored overnight at 4°C to allow the hydration.

Emulsions were prepared following a modified method of Dickinson & James (2000) consisting of 20 vol% tetradecane, 0.5 wt% whey protein, 0.2 wt% pectin, and premixed for 4 minute on ice using a PRO300A homogenizer (Pro Scientific Inc., Oxford, Ct). The pH was then adjusted to 4.2 with diluted NaOH.

The mixture was homogenized in 3 passes at 40 MPa in a high pressure homogenizer (Emulsi-Flex C5, Avestin Inc, Ottawa, ON, CA) on ice. Several control emulsions were made featuring the control pectin and protein (CP + Protein), control pectin and no protein (CP + No protein), and just protein (No pectin). The emulsion control samples will be referred to in this paper by the codes shown above in parenthesis.

## 2.5 Emulsion Evaluation

#### 2.5.1 Emulsion activity

Emulsion activity (%EA) was measured according to the method used by Mishra, Mann, & Joshi (2001) with modifications by centrifuging each sample in a graduated tube at room temperature for 5 minutes at 2600g using a Fisher Scientific Marathon 3200 centrifuge. The samples were held at 20°C and %EA was measured on days 2,8,16, and 24. % EA was calculated using the following formula:

EA (%) = (the volume of the emulsion phase/ total volume of the system) x 100

#### 2.5.2 Particle Size

The particle size of the emulsions was measured on a Particle Size Analyzer (90 Plus, Brookhaven Inst., Holtsville, NY) with a 50 mV diode laser (90° angle) and a BI-9000AT correlator. The effective diameter of the particles in the emulsions was calculated from a collective fit of the intensity autocorrelation function acquired from the intensity fluctuation of the scattered light with 90-Plus particle sizing software (version 3.37, Brookhaven Instruments, Worcestershire, UK). The emulsions were diluted with a 1:1,000 dilution factor which was a similar dilution factor used by Einhorn-Stoll, Glasenapp, & Kunzek (1996). Additionally, this dilution factor was verified based on preliminary data. Several dilution factors were tested on a test emulsion sample and 1:1,000 dilution factor provided the most accurate measurement with the smallest standard deviation (data not shown).

## 2.5.3 Rheology

The viscoelastic properties of the emulsions were measured on the dispersed phase using a Rheometric Scientific SR5000 Controlled Stress Dynamic Rheometer<sup>TM</sup> (Rheometrics, Piscataway, New Jersey, U.S.A.) outfitted with a cone and plate apparatus (60 mm diameter, 0.0385°, 0.5 mm gap). The emulsions were stored at 20°C and rheology measurements were taken on days 3-4, 8-9, and 15-16. A dynamic strain test was conducted with 0.1% to 100% strain with a 1 Hz frequency. A frequency sweep test was also run from the linear viscoelastic region as determined from the strain test. The frequency sweep test was run from 10<sup>-3</sup> to 10 Hz.

#### 2.5.4 Turbiscan

A TurbiSoft Classic MA2000 (Formulaction, Toulouse, France) system was used to measure the particle distribution in the emulsions over time. Flat bottom test tubes were filled with 7 mL of emulsion and stored at  $4^{\circ}$ ,  $20^{\circ}$ , or  $40^{\circ}$ C. The tubes were scanned at regular intervals over the course of 26 days. The backscattering (BS) and transmission profiles were collected on each sampling. To evaluate the samples, a reference plot was made by subtracting the initial BS measurement from all subsequent BS measurements, resulting in a plot that represents the change in the sample over time.

The destabilization kinetic for the clarification front was calculated at -40% BS on the relative BS plot using a modified method of Mengual, Meunier, Cayre et al (1999) to determine the settling rate of emulsions. To do this, the area of the tube where -40% BS occurred in the clarification front at all measured time points in the BS were plotted against the elapsed time. *2.6 Statistics and Replications* 

All statistics were run on SAS version 14.2. All emulsion samples were formulated in experimental duplicates.

#### **3. Results and Discussion**

#### 3.1 Emulsion Activity (EA)

EA were reported in **Figure 1** and showed changes over the course of 26 days. The emulsions formulated with modified pectin showed a large decrease of EA from day 2 to day 8 (~30%) and then showed only a small decrease on day 16 and 24. The emulsions formulated from unmodified pectin showed a continually gradual lowering of %EA (< 10% from day 2 to day 8). The no pectin sample had a fast decrease in EA (~30% between day 2 and 8). A two way random effect model returned a p-value of < 0.0001 showing that all 7 samples are significantly different. However, a two way random effect model featuring just the 4 emulsions

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formulated with modified pectins suggests that these four emulsion are not significantly different (p-value = 0.7398). The tests returned the different results because they compare the value of each sample to the average of the entire population. The average of the population was greatly changed based on whether the 7 samples were averaged or just the 4.

#### 3.2 Particle Size

The particle size of the emulsions were measured and reported in **Figure 2**. All emulsion samples were significantly different (p-value= 0.0062). There was no observable trend in the particle size numbers. The emulsion featuring the smallest particle size was the emulsion formulated with CP and no protein ( $848 \pm 41 \text{ nm}$ ) followed by the emulsion formulated with pectin modified from 13 kDa isozyme ( $1060 \pm 10 \text{ nm}$ ), the emulsion formulated with CP + Protein ( $1100 \pm 112 \text{ nm}$ ), the emulsion formulated with pectin modified by 27 kDa isozyme ( $1141 \pm 125 \text{ nm}$ ), the emulsion formulated with just protein ( $1208 \pm 132 \text{ nm}$ ), the emulsion formulated with pectin modified by 36,27, and 13 kDa isozyme ( $1274 \pm 147 \text{ nm}$ ), and then the emulsion formulated with pectin modified by 36 kDa isozyme ( $1569 \pm 104$ ).

#### 3.3 Rheology Results

The results of the dynamic strain test are shown in **Figure 3a-3b.** The figures show that the G' of the samples decreases over time which indicates instability in the samples. The G' for the emulsion formulated with pectin modified by 36 kDa isozyme and the emulsion formulated with pectin modified by the 27 kDa isozyme feature roughly the same G' value. The emulsion formulated with pectin modified by the 13 kDa isozyme and the emulsion formulated with pectin modified by the combination of 36, 27, and 13 kDa isozyme have a lower G' than the emulsions mention above and the two emulsions feature the same shaped curve. This suggests the behavior of the pectin modified by the combination of 36, 27, and 13 kDa isozyme is dominated by the 13

kDa isozyme. The emulsion formulated with just protein appears to have the highest G' but this is likely due to the larger aqueous phase, so there is less water in the cream layer being measured by the rheometer. The true drop in G' of this emulsion would be extreme but since we are only measuring the G' of the dispersed phase, this drop is not observed. The large drop in aqueous phase is observable with the human eye as seen in **Figure** 7. The greatest change in the samples appears to happen between days 3/4 and 8/9. The drop between days 8/9 and days 15/16 is much smaller. The frequency sweep did not provide any useful information on the emulsion samples because the limit of detection for rheometer instrument was reached.

### 3.4 Turbiscan Results

All emulsions showedcreaming and phase separation (**Figures 4a and 4b**). The creaming phenomenon occurs when the dispersed phase has a lower density than the continuous phase. This results in the emulsion phase compacting in size and rising to the top of the emulsion as seen in the pictures of the emulsions used for the Turbiscan measurements (**Figure 7-8**). **Figure 4a** highlights several of the important elements of the Turbiscan BS profile. The Y-axis measures the BS intensity in percent, comparative to an external standard (suspension of polystyrene latex beads; particle diameter  $d= 0.3 \mu m$  and a volume fraction  $\phi= 10\%$ ) and the X-axis presents the sample height in mm (h=0 mm is equal to the bottom of the test tube). In order to picture the change in the sample over time, the BS profile measured at time t=0 has been subtracted from all the other measurements resulting in a BS plot relative to the initial measurement at t=0. As a result, the first measurement at t=0 is on the line BS= 0%.

In all of the emulsion samples, the oil droplets migrate from the bottom of the tube to the top which yields a gradual drop in concentration at the bottom of the sample. This region of the sample is known as the clarification front; this is the area at the bottom of the tube that results in

a decrease in BS (negative peak) where the emulsion dispersed phase has started to move up the tube due to it having less density than the continuous phase of the emulsion (labeled in **Figure 4a**). This migration results in a steady increase in concentration at the top of the sample known as cream formation and results in a spike in the BS representing the disperse phase collecting and compacting at the top of the tube (labeled in **Figure 4a**). The middle area of the spectrum shows the change in particle size in the emulsion over time.

The Turbiscan plots shown in **Figure 4a-4b** show a large difference between an emulsion sample formulated with modified pectin and one formulated with unmodified, control pectin. The emulsion formulated with modified pectin (**Figure 4a**) shows a non-linear phase separation as the emulsion separates rapidly in the first 100 hours and then slows down and separates at a slow pace. The emulsion formulated with control pectin (**Figure 4b**) shows a steady linear separation over the course of the entire measurement period. It is also important to note that the modified pectin sample separated less over the time period (the clarification front moved about 5mm over the course of the experiment at  $40^{\circ}$ C) than the control pectin (the clarification front moved 35 mm over the course of the experiment at  $40^{\circ}$ C).

The data in **Figures 5a-5g** shows the destabilization kinetics for the clarification front for the emulsions stored at the 4 °C, 20 °C, or 40 °C. The emulsions formulated with modified pectins show a first order destabilization kinetic (**Figure 5a-5d**) while the emulsions formulated with unmodified control pectins (**Figure 5e-5g**) show a zero order destabilization kinetic, meaning that the rate of loss of clarification front is steady throughout the storage period and is independent of the concentration (Mann and Jones 2000). Also evident in these plots is that as the storage temperature increases, the rate of clarification increases. To further compare between modified pectin samples, a best fit power law was fitted to the destabilization plots to obtain clarification rates. The rates of clarification in the samples are shown in **Figure 6a** at the three different temperatures. The figure shows that the emulsion formulated with 13 kDa isozyme has the slowest separation and that there is not much difference between the rates of the other emulsions. The rates of the control emulsion samples are shown in **Figure 6b**. The emulsion formulated with CP + protein gave the slowest clarification rate ( $4^{\circ}C = 8.05 \pm 1.8 \mu m/hr$ ,  $20^{\circ}C = 18.0 \pm 3.3 \mu m/hr$ ,  $40^{\circ}C = 53.0 \pm 2.8 \mu m/hr$ ), followed by the emulsion formulated with CP + No Protein ( $4^{\circ}C = 16 \mu m \pm 2.1 \mu m/hr$ ,  $20^{\circ}C = 27 \pm 2.1 \mu m/hr$ ,  $40^{\circ}C = 57.0 \pm 2.8 \mu m/hr$ ), and then the emulsion formulated with no pectin ( $4^{\circ}C = 34 \pm 2.1 \mu m/hr$ ,  $20^{\circ}C = 57.0 \pm 42 \mu m/hr$ ,  $40^{\circ}C = 83.0 \mu m/hr$ ).

## 4. Conclusions

The emulsions made from modified pectins featured a first order reduction of stability while the emulsions made from unmodified pectin and just protein featured zero order reductions. In a previous study, the value of  $\zeta$ -potential was measured as way to estimate the charge of the pectin on the shear plane(Rivner and Wicker 2008). That study showed that the range of charge on the modified pectin was from -39 mV to -45 mV while the control pectin had a charge of -12 mV. This suggests that there is excess negative charge on the modified pectin for there to be a synergistic effect with the protein.

Furthermore, there were no visible differences across the tests between charged modified pectin samples. This study further shows that the %DE range these modified pectin are in is "a dead zone" in terms of increase emulsion stability. This is in accordance with a previous study by Lee, Rivner, Urbauer, Garti & Wicker (2008) that showed "a dead zone" in modified pectin in this %DE range in terms of calcium sensitivity.

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Figure 5.1. The EA of the various emulsion samples measured using a modified version of the method by Mishra et al. (2001). The EA was measured at day 2, 8, 16, and 24. The samples were stored at 20°C. The pectin used in the emulsions were created using the crude enzyme that contained 36, 27, and 13 kDa isozymes, SPbHPb9 enzyme that contains 36 kDa isozyme, SPuHPu enzyme that contains 27 kDa isozyme and SPbQu enzyme that contains 13 kDa isozyme. These emulsion are refered to by the isozymes used to modify the pectin. Additionally, several control emulsions were made featuring the control pectin and protein (CP + No protein), and just protein (No pectin).



Figure 5.2. The particle size of the emulsion samples measured on day 2 at 4  $^{\circ}$ C.



Figure 5.3a. The G' values of all the emulsion samples at day 3-4 stored at 20°C. The dynamic strain test was conducted with 0.1% strain to 100% strain with a 1 Hz frequency.



Figure 5.3b. The G' values of all the samples at day 8-9 stored at 20°C. The dynamic strain test was conducted with 0.1% strain to 100% strain with a 1 Hz frequency.



Figure 5.3c. The G' values of all the samples at day 15-16 stored at 20°C. The dynamic strain test was conducted with 0.1% strain to 100% strain with a 1 Hz frequency.



Figure 5.4a. Turbiscan measurements for emulsions formulated with pectin modified with 27 kDa isozyme combination held at 4°C, 20°C, and 40°C. The separation of the phases occurred in an un-linear fashion.



Figure 5.4b. Turbiscan measurements for emulsions formulated with control pectin held at 4°C,

20°C, and 40°C. The separation of this emulsion occurred in a linear fashion.



Figure 5.5a. The destabilization kinetics for the emulsion formulated with pectin modified by 36,27, and 13 kDa isozyme. The destabilization kinetic is first order. Each emulsion sample was run in duplicate and the replications are labeled "a" and "b".



Figure 5.5b. The destabilization kinetics for the emulsion formulated with pectin modified by 27 kDa isozyme. The destabilization kinetic is first order. Each emulsion sample was run in duplicate and the replications are labeled "a" and "b".



Figure 5.5c. The destabilization kinetics for the emulsion formulated with pectin modified by 36 kDa isozyme. The destabilization kinetic is first order. Each emulsion sample was run in duplicate and the replications are labeled "a" and "b".



Figure 5.5d. The destabilization kinetics for the emulsion formulated with pectin modified by 13 kDa isozyme. The destabilization kinetic is first order. Each emulsion sample was run in duplicate and the replications are labeled "a" and "b".



Figure 5.5e. The destabilization kinetics for the emulsion formulated with CP + Protein. The destabilization kinetic is zero order. Each emulsion sample was run in duplicate and the replications are labeled "a" and "b".



Figure 5.5f. The destabilization kinetics for the emulsion formulated with CP + No Protein. The

destabilization kinetic is zero order. Each emulsion sample was run in duplicate and the replications are labeled "a" and "b".



Figure 5.5g. The destabilization kinetics for the emulsion formulated with No Pectin. The

destabilization kinetic is zero order. Each emulsion sample was run in duplicate and the replications are labeled "a" and "b".



Figure 5.6a. The slope of the power law of the emulsion samples formulated with modified pectins stored at  $4^{\circ}$ C,  $20^{\circ}$ C, and  $40^{\circ}$ C measured on the Turbiscan instrument.



Figure 5.6b. The rate of the clarification front of the emulsion samples formulated with control pectin stored at 4°C, 20°C, and 40°C measured on the Turbiscan instrument.



Figure 5.7. Emulsion samples made from pectins modified with 36, 27, and 13 kDa separated isozymes and control pectin samples. The emulsions were held at 40 °C and the tubes are shown

on day 1, 8, 17, and 26. These emulsion tubes were scanned in the Turbiscan instrument to produce the BS spectra in **Figures 5a-5h**.



Figure 5.8. Emulsion samples made from pectins modified with 36, 27, and 13 kDa separated isozymes and control pectin samples. They emulsions were stored for 30 days at 4°C, 24°C, 40°C, and 62°C. These emulsion tubes were scanned in the Turbiscan instrument to produce the BS spectra in **Figures 5a-5h**.

# CHAPTER 6

## CONCLUSIONS

The information in chapter 3 describes that the individual isozymes of PME can be eluted individually using multiple separations with IEX chromatography. The process was hindered by likely formation of a PME-pectin complex. The isozymes had differing characteristics and partial amino acid sequences. The PME isozymes yielded charged modified pectin with differing distribution patterns of charge along the pectin backbone. Combinations of multiple isozymes left larger blocks of continuous charge than individual isozymes.

The data in chapter 4 depicts that both the %DE and the distribution of DE played an important role in determining the overall functionality of the pectin (as measured by the calcium sensitivity test). The larger the blocks of continuous charge on the pectin backbone, the greater effect the blocks had on the functionality of the pectin. This was evident from the NMR frequency of GGG/GGE having a much greater correlation with G' then the GG frequency. However, since the relationship was best described with a power function (as opposed to a linear trend), it is hard to determine how much an increase in block charges really affects the overall functionality of the pectin.

There were no visible differences across the tests in chapter 5 between charged modified pectin samples. The emulsions made from modified pectins featured a first order reduction of stability while the emulsions made from unmodified pectin and just protein featured zero order reductions. In a previous study, the value of  $\zeta$ -potential was measured as way to estimate the charge of the pectin on the shear plane(Rivner & Wicker, 2008). That study showed that the

range of charge on the modified pectin was from -39 mV to -45 mV while the control pectin had a charge of -12 mV. This suggests that there is excess negative charge on the modified pectin for there to be a synergistic effect with the protein.

Furthermore, there were no visible differences across the tests between charged modified pectin samples. This study further shows that the %DE range these modified pectin are in is "a dead zone" in terms of increase emulsion stability. This is in accordance with a previous study by Lee, Rivner, Urbauer, Garti & Wicker (2008) that showed "a dead zone" in modified pectin in this %DE range in terms of calcium sensitivity.