VALENCIA PECTINMETHYLESTERASE ISOZYMES RESULT IN PECTINS OF UNIQUE CHARGE DOMAIN AND FUNCTIONALITY

by

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(Under the Direction of Louise Wicker)

ABSTRACT

Based on the hypothesis that PME fraction containing 36/27 kDa peptides (U-PME) will yield differently modified pectin than PME fraction containing 36/13 kDa peptides (B-PME), the objective of this study was to isolate the Valencia PME isozymes, de-esterify pectin to a target DE and characterize the resultant pectin products for charge and charge distribution. In addition, the calcium sensitivity was estimated by viscosity and gelling properties with $CaCl_2$, as well by ζ-potential, an indicator of the surface charge. Finally, interactions of individual caseins with modified pectins was compared by sedimentation, protein/pectin content, particle size and ζ -potential.

Valencia PMEs de-esterified pectin to 63% (B-Pec) and 61%DE (U-Pec) from 73%DE (O-Pec), did not decrease molecular weight, created more negative ζ-potential, and had different charge distributions. Based on elution from ion exchange chromatography (IEX), chemical shifts in NMR spectra, and ζ-potential, we observed a blockwise de-esterification pattern following a 10% decrease in DE. From elution profile of IEX, the peak of B-Pec and U-Pec widened and shifted to a higher ionic strength compared to O-Pec. Finally, we concluded that B-PME and U-PME had different action patterns based on the 2-fold increase in the frequency of

contiguous carboxylic acid groups (F_{GGG}). Also, we concluded that U-Pec had less contiguous blocks of carboxylic acid groups than B-Pec but a greater population of pectin molecules were modified by U-PME.

In the presence of 35 mM $CaCl_2$, 2% B-Pec and U-Pec formed a gel, in contrast to O-Pec. B-Pec and U-Pec were 20 or 50-fold higher G' (elastic element) than O-Pec with $CaCl_2$ while O-Pec had higher viscosity than B-Pec or U-Pec without $CaCl_2$.

From the interaction of individual caseins with pectins, addition of pectin to the milk fractions had unique effect and ranged from increasing the extent of sedimentation to minimizing sedimentation at pH 3.8. κ-Casein had no precipitate initially, but addition of pectin resulted in sedimentation, and U-Pec resulted in the greatest precipitate. Based on particle size, ζ-potential, and viscosity, U-Pec or B-Pec had more effect on milk dispersions than O-Pec. β-Casein seemed to interact with pectins more like κ-casein than $\alpha S_{1,2}$-casein.

INDEX WORDS: Pectinmethylesterase, Modified pectin, High methoxyl pectin, Degree of esterification, Charge and charge distribution, NMR, ζ -potential Calcium sensitivity, Casein fractions,

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DEDICATION

I would like to dedicate this dissertation to my family for all of their love and support.

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CHAPTER 1

INTRODUCTION

Pectins are used for gelling and thickening in food applications. Several studies reported novel pectin usages, like biodegradable water-soluble films, bulking agents, coating agents, chelators, emulsifiers, viscosity modifiers and stablilizers (Christensen and others, 2001; Laurent and Boulenguer, 2003; Voragen and others, 1995). High methoxyl pectin (HMP) is used as a stabilizer in acid milk drinks, like fruit milk drinks, yogurt drinks, soymilk, butter-milk, whey drinks, and kefir. These products are subject to gelling, serum separation and sedimentation. Development of new pectin-stabilized dairy products is founded on understanding how stabilizers interact with other dairy components to prevent quality defects. Understanding mechanisms of interactions of pectins with food components is enhanced by specific information on structure and function of pectin. Pectin structure can be enzymatically changed to create tailored pectins with unique charge properties.

Pectin methylesterase (PME, E.C. 3.1.1.11) catalyses the demethoxylation of esterified pectins. Plant PMEs de-esterify HM pectin resulting in low methoxyl pectin (LMP), yielding block-structures on the homogalacturonan backbone, and allowing $CaCl_2$ cross linking of pectin chains of HMP without sugar (Hotchkiss and others, 2002).

In this study, the overall objective is to modify commercial HMP using citrus PMEs to prepare tailored, calcium sensitive HMP and characterize physico-chemical properties. In the third chapter, we studied the pattern of modification by two Valencia PME isozymes

on the chemical structures of original, two modified HMP, and fractions of original and modified pectins after preparative ion exchange chromatography (IEX). The pectins were analyzed for molecular weight by multi-angle light scattering, pattern of elution from IEX, charge distribution by NMR, and charge by zeta (ζ)-potential. In the fourth chapter, the calcium sensitivity of the two modified pectins in the presence of $CaCl_2$ was investigated and compared with original pectin. These studies were conducted in dilute solutions by the determination of viscoelastic properties (G' and G") and ζ-potential and on calcium gels by measurements of the gel strength. In the fifth chapter, the potential of modified pectin as a stabilizer was evaluated in model systems of purified milk casein fractions. Interactions of original and modified pectins and casein in an acidified milk system were quantified by sedimentation, protein/pectin content, particle size and ζ -potential. The dispersion systems included non-fat milk and casein fractions, α $S_{1,2}$-, β-, and κ-casein, in acetate buffer, pH 3.8. Especially, κ-casein was further investigated by studying the microstructure, viscosity, and peptide profile of the mixed system with two different modified HMPs.

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CHAPTER 2

LITERATURE REVIEW

Pectin Structure

The composition of pectin varies with source, conditions of extraction, location, and other environmental factors (Daas and others 2001). Pectin is an anionic polysaccharide consisting of a linear backbone of α (1-4)-D-galacturonic acid residues, with periodic interruptions of 1,2 linked L-rhamnose residue. Other neutral sugars are present as side chains which makes the backbone irregular. The homogalacturonan portions of the polymer are referred to as the 'smooth' regions, while the rhamnose-rich zones are called 'hairy' regions, as the sugars carry neutral oligosaccharides side chains. A considerable proportion of the galacturonic acid residues of the backbone are methyl-esterified (Voragen and others 1995; De Silva and others 1995). According to their degree of esterification (DE), pectins are classified as high-methoxyl pectin (HMP, > 50% DE) and low-methoxyl pectin (LMP, < 50% DE). Even with similar degrees of methyl esterification, different methyl ester distributions can confer different functionalities in pectins. In general, two different types of methyl ester distributions can be discerned: intra- and intermolecular. For determination of intramolecular distribution (distribution of methyl esters within a pectin polymer), NMR or molecular degradation studies are required, whereas, for intermolecular distribution (the distribution of substituents over various pectin polymers in a mixture), chemical and enzymatic techniques using fractionation must be applied in investigation (De Vries and others, 1986; Daas and others, 2001).

The primary structure of the pectic polymers has been determined by extraction with chelating agents, followed by enzymatic hydrolysis (endo-polygalacturonase) and mass spectrometer analysis of the resulting oligomers as well as ^{13}C and ^{1}H NMR spectroscopy (De Vries and others, 1982; Pėrez and others, 2000; Limberg and others, 2000). Recently, a variety of experimental probes of higher-level pectin structures have been reported for samples in both the solid-state and liquid-state, using a diversity of techniques. Examples of the former include transmission electron microscopy (Fishman and others, 1993), fiber diffraction (Walkinshaw and Arnott, 1981), atomic force microscopy (Kiby and others, 1996), and ^{13}C NMR (Jarvis and Apperley, 1995). Examples of the latter consist of light scattering (Ousalem and others, 1993), viscosimetry (Cesaro and others, 1982; Axelos and others, 1987; Harding and others, 1991), ^{13}CNMR (Catoire and others, 1997), potentiometry and circular dichroism (Ravanat and Rinaudo, 1980), small-angle neutron scattering (Cros and others, 1996), and small-angle X-ray scattering (Axelos and others, 1996).

Pectin Methylesterase

Pectin methylesterase (PME, E.C. 3.1.1.11) is the enzyme that catalyses the demethoxylation of esterified pectins. They have been isolated from various sources and with differing action patterns with respect to the removal of methyl esters. Three mechanisms are classically considered for the action pattern of PMEs (Grenwood and Milne, 1968): (1) a single-chain mechanism, where the binding of the enzyme is followed by a conversion of all contiguous substrate sites on the homogalacturonan; (2) a multi-chain mechanism, where the enzyme-substrate complex dissociates after each reaction, resulting

in deesterification of only one residue for each attack; (3) a multi- attack mechanism, in which the enzyme catalyses the deesterification of a limited number of residues for every active enzyme complex formed. For identification of mechanisms of action of PME on pectins, there are two different types of methods: indirect methods (enzyme degradation method, study of calcium binding on pectin, ion-exchange chromatography method) and direct methods (^{1}H NMR and ^{13}C NMR spectroscopy).

Acidic microbial (*Aspergillus japonicus, Aspergillus niger, Aspergillus foetidus*) PMEs are claimed to lead to pectins with a random distribution of free carboxyl groups (multiple chain mechanism) (Ralet and others 2001b; Thibault and Rinaudo 1985). Pectins de-esterified by fungal PMEs are able to cross-link via calcium bridges, yielding DE values below 30–35 % (Ralet and others 2001b). The action of alkaline PMEs from higher plants (banana, tomato, orange, apple, strawberry) and from fungi (*Trichoderma reesei*) is thought to catalyze the demethylation of pectin linearly along the chain (single chain mechanism) and to result in blockwise arrangement of free carboxyl groups in the pectin molecules. The de-esterification starts near the free carboxyl groups or from the reducing end of the pectin chain. This gives rise to the introduction of so-called block-structures of adjacent free galacturonic acid units on the HG backbone, which allows calcium cross-linking of pectin chains (Christensen and others 1998; Limberg and others, 2000; Hunter 2002, Ly-Nquyen and others 2002; Savary and others 2002). Some plant PMEs appear to have the capacity to remove a limited number of methyl esters per reaction, giving rise to short, un-esterified blocks (Willats and others 2001). In a study on apple PME by Denès and others (2000b), the action patterns at pH 7.5 consisted of a blockwise distribution by a single

chain mechanism, while the action at pH 4.5 was also a blockwise distribution, but with shorter blocks on multiple chains.

Valencia PME

Multiple PME isozymes are present in orange peel, and individual isozymes can be distinguished by their expression patterns as well as by their physical and biochemical properties (Bordenave, 1996). Christensen and others (1998) isolated salt-dependent and salt-independent isozymes from navel orange peel, and Versteeg and others (1980) also isolated two major PME isozymes in Navel orange. In Navel orange, thermostabile (TS-) PME was capable of rapidly clarifying the juice, while the two thermolabile (TL-) PMEs destabilized the cloud very slowly or not at all. In Valencia orange, four PME activity peaks were resolved by Heparin-Sepharose chromatography (Cameron and others 1998). One of four PME isozymes did not affect orange juice cloud, while the other three isozymes destabilized orange juice cloud at varying rates. Wicker and others (2002) demonstrated clarification of citrus juices by TL-PME from Valencia pulp in the presence of cations. Another study by Ackerley and Wicker (2003) mentioned that TL-PME containing 36kDa and 27kDa peptides clarified juice faster than TL-PME containing 36kDa and 13kDa peptides. In a study by Savary and others (2002), individual Valencia PME isozymes were isolated. A following study by Hotchkiss and others (2002) demonstrated that salt-independent orange PME modified pectin produced calcium-sensitive pectin while preserving its molecular weight. Hunter (2002) also mentioned observing no molecular weight change in Valencia pulp modified pectin after separation on a Hi-Trap SP and heparin column.

Pectin Fractionation

Before analyzing the specific characteristics of pectin, some preparations are required depending on its usages even if commercial pectins are used. The methods of extraction, preparation, and purification will affect some degree of fraction of the large number of different compounds. For purifying pectins, a few different methods have been employed. For instance, sugars, acids, metallic ions, and many ash constituents can be removed for the most part by successive precipitations with acid ethanol (alcohol precipitation). However, some of the accompanying hemicellulose and starch, as well as some cations, attached to the carboxyl groups of the pectin requires special treatment of the solutions. Dialysis, ion exchange chromatography (IEX), and metal precipitation are also used for purification of pectin (Fishman, 1991; Kravtchenko and others, 1992b). Separation and fractionation by high-performance anion-exchange chromatography (HPAEC) or size exclusion chromatography (SEC) have been successfully performed. SEC is a well-known method of separating substances that differ in molecular size (Kravtchenko and others, 1992b). Since the molecular size distribution of a polymer can be important in understanding its functional behavior, SEC has often been used to fractionate pectin preparations (Rombouts and Thibault, 1986; Hourder and Muller, 1987). In the case of IEX, the underlying principle is that separation is achieved according to the charges of pectin molecules, which depend on the number of dissociated carboxyl groups present on the individual molecules. Basically, pectins can thus be fractionated by IEX according to their degree of methoxylation (1°) as well as their covalently linked neutral sugar content (2°).

Pectin Characterization

Many of the functionalities of pectins are related to their chemical structure. The relevant parameters include the amount of galacturonic acid and neutral sugars, the amount and distribution of substituents (methoxyl and acetyl groups), and the molecular weight (Axelos and Thibault, 1991).

Degree of Esterification

The degree of esterification (DE) is defined as the moles of methoxyl groups per 100 moles of galacturonic acid residues. The DE of pectin is usually between 60 and 90%, depending upon the species, tissues, and maturity of plants, whereas commercial pectin usually has DE values between 30 and 76% (Jiang and others, 2001). For instance, vegetable pectins from cucumbers, celery, tomato and paprika have their maximum DE within an esterification with randomly range of 50 – 60%, and the carboxyl groups are randomly distributed in all these preparations. However, vegetable pectins from cauliflower, radishes, and carrots have, a fraction of pectin molecules which have blocks of free carboxyl groups as well as a pectin fraction with randomly distributed carboxyl groups. Moreover, the latter fraction may constitute up to 50% of the pectin (Anger and Dongowski, 1985).

Within pectic fractions, there exist various DE values. Anger and Dongowski (1984) demonstrated the occurrence of various fractions of pectin molecules with different degree of methylation. Specifically, mechanically degraded pectins of similar degrees of methoxylation eluted earlier as the molecular size decreased (Anger and other 1977). Kravtchenko and others (1992b) found that the DE of the IEX fractionated pectins

decreased regularly in later eluting. Moreover, fractions with a DE very different from that expected had a higher content of phenolics. This was especially true for the last two fractions from the three pectin samples. Hunter (2002) also showed the same results in that there was decrease in DE with an increase in fraction number using FT-IR measurement. Hourdet and Muller (1987) reported that the DE was decreased with an increase in fraction obtained by SEC. Specifically, the ester type carboxyl groups decreased, while the total anhydrogalacturonic acid and free carboxylic groups increased from fractions 1-4. Ralet and others (2001a) suggested that the high DE values and less charge-dense molecules eluted first, with a decrease in %DE as elution volume from IEX increased. Kravtchenko and others (1992a) also agreed that structural features other than average DE govern the strength of binding to IEX.

Several procedures have been reported for determination of DE in pectin such as gas-liquid chromatography (GLC), high performance liquid chromatography (HPLC), enzymatic methods, and titration or colorimetry method (Cochrance, 1975; Banjongsinsiri and others, 2003). However, HPLC methods are simple and widely used, but a rather high amount of pectin is necessary (around 30 mg), partly because an external rather than an internal standard is used. Moreover, just before the methanol peak, the presence of a negative peak in the HPLC may lead to errors in the integration of the methanol peak (Levigne and others, 2002). Chemical procedures for the DE determination require less material (100 μg), but they are time consuming since they require the reduction of the esterified galacturonic acid residues before methanolysis, derivation, and GLC analysis. An alternative methodology is colorimetry, which requires more material (2 or 3 mg)

(Mannes and others, 1990). Some direct determination of DE has been proposed by ^{1}H NMR (Andersen and others 1995; Denės and others, 2000a) and by FT-IR (Gnanasambandam and Proctor, 2000; Hunter, 2002). ^{1}H NMR analysis, which is widely used for structural investigation of polysaccharides, was suitable for the determination of pectin DE. In the ^{1}H NMR spectra of pectin, chemical shifts (δ) of protons of E (esterified galacturonic acid) and G (de-esterified galacturonic acid) at H-4 are used for determination of %DE. NMR method allows in only few minutes an accurate simultaneous qualification of DE from a few mg of pectin, without the need of external standards (Bėdouet and others, 2003). Moreover, compared with the titration method, it requires only a small amount of material, and no differences between DE values calculated by NMR compared to those calculated by titration have been demonstrated (Grasdalen and others, 1988; Rosenbohm and others, 2003). All studies were performed at 80°C because the boiling point of methanol is 64.9°C. This was considered as a possible limit for the determination of the pectin DE by ^{1}H NMR spectroscopy. Thus, analysis was performed at a higher temperature (Bėdouet and others, 2003).

Molecular Weight

Pectins are both polymolecular and polydisperse, exhibiting significant heterogeneity with respect to both chemical structure and molecular mass. Accurate determination of molecular mass and molecular mass distribution of pectin still remains a challenge because of the heterogeneous nature of pectin, such as the presence of smooth and hairy regions and the varying inter- and intramolecular distribution of methyl esters. Kravtchenko and others (1992b) examined three industrial pectin preparations via SEC and

light scattering. Fractions of a given hydrodynamic volume within one pectin sample remained highly heterogeneous on the basis of their molecular weight (MW), indicating the coexistence in industrial pectins of particles of very different shapes. However, molecular masses in the range of 1000 kDa may be representative of many pectins (Voragen and others 1995; Serge Pėrez and others, 2000; Daas and others 2001). Depending on modification, there is a different change in MW. Namely, pectin de-esterified by a salt-independent orange PME showed no significant decrease in MW, while pectin treated with alkali resulted in a decrease in Mw. Hotchkiss and others (2002) also found that PME modified pectin preserved its molecular weight using HPSEC and MALLS.

In previous studies, gel permeation chromatography, high performance size exclusion chromatography (HPSEC), low angle laser light scattering (LALLS), and multi-angle laser light scattering (MALLS) have been widely used for examination of Mw.

Methylester Distribution and Charge Density

The total charge and distribution of charge in pectins affect the function of commercial pectin, such as a stabilizing agent in juices and juice beverages as well as the stability of acidified protein drinks (Glahn, 1982; Christensen and others, 1997; Wicker and others, 2003). In addition, charge has an impact on gelling properties and calcium binding potential (Limberg and others, 2000; Ralet and others, 2001b; Willats and other, 2001). The methylester distribution of pectin is very complex due to the fact that the methylester distribution should be revealed on an intramolecular level (within one molecule) and on an intermolecular level (distribution of methylester over various pectin molecules within a mixture) (De Vries and others, 1983; De Vries and others, 1984). For determination of

intramolecular distribution, NMR or molecular degradation studies are required, whereas for intermolecular distribution, chemical and enzymatic techniques through fractionation must be applied in investigation (De Vries and others, 1986; Daas and others, 2001).

The combination of SEC separation by the molecular weight distribution and computer studies was performed to determine the distributions expected for randomly methyl esterified samples of varying DE (Tuerena and others, 1981; Tuerena and others, 1984). However, because the SEC technique employed was not able to fully separate the various oligomers produced, the methyl ester distribution could not be resolved in great detail. The distribution of methoxyl groups and charge density has been studied by IEX, using gradient elution. Basically, pectins can be separated by IEX according to the charges of pectin molecules, which depend on the number of dissociated carboxyl groups present on the individual molecules (Heri and others, 1961). The pectin binds to the anion exchanger by electrostatic forces between the surface charges of the pectin and the dense clusters of charge groups on the exchangers (Scopes, 1994).

As observed by IEX (Kravtchenko and others, 1992b), standard pectins eluted in a rather narrow peak, with their elution time increasing with decreasing DE. The broadness of peaks indicated that pectin molecules were distributed over a wide range of DE. In addition, they found that larger blocks were found in the enzyme de-esterified pectin as compared with the alkali and acid de-esterified material. In elution patterns of IEX, Schols and others (1980) found that pectins with a random distribution of methoxyl groups eluted in a peak proportional to DE, with narrow distribution curves. However, the elution of citrus PME demethylated pectins, which gave the blockwise action, displayed a wider peak,

which can be explained by the fact that the separation was based on the charge density over the molecules and not on the total charge of the molecules. Anger and Dongowski (1984) also mentioned the difference in the distribution of the free carboxyl groups along the pectin backbone in IEX elution. A blockwise distribution might result in zones of higher charge density, which bind strongly to the ion exchanger. Therefore, from this information, it can be concluded that the de-esterification methods by Valencia PME produce the modified pectins containing a small change in the DE, but a completely different pattern towards IEX because of the change of charge density. Otherwise, the size of the pectin molecules also affects the elution pattern. Namely, mechanically degraded pectins of similar degrees of DE elute earlier as the molecular size decreases (Heri and others, 1961; Anger and others, 1977).

^{1}H NMR spectroscopy has proven itself a valuable tool in the study of pectins. In a study by Andersen and others (1995), ^{1}H NMR spectra are shown that differentiate between dyads, triads, and some tetrads in partly esterified galacturonic acid, yielding unique information about the sequential structure. They suggested that NMR spectroscopy could be used as a direct assay for PME activity, measuring and characterizing the block de-esterification. Denės and others (2000) described the behavior of purified apple PME at pH 7.0 and 4.5 by a combination of indirect (IEX) and direct (^{1}H NMR spectra) methods. They suggested the frequency of F_{GGG} and F_{EEE} as a function of final DE following the action of PME, and that the average number of successive E residues should be considered as equal to the degree of multiple attacks of PME. Here, E and G mean esterified unit and de-esterified one, respectively. In addition, the frequencies of F_{GGG} which were higher

than the Bernouillian probabilities were considered as the blockwise distribution, when the DE decreased. Grasdalen and others (1996) also reported that the enzymatic reaction resulted in a high content of homogeneous triads (GGG and EEE), and demonstrated the production of a sequential structure. Andersen and others (1995) mentioned that a block-type distribution in the enzyme treated samples was indicated by stronger lines in the spectra, corresponding to contiguous arrangements of esterified and de-esterified units denoted by EE, EEE and GG, as well as corresponding weaker lines from residues characterizing block transitions, EG and GE.

Pectin Gelation

The most attractive property of pectin for industrial applications is its gelling activity. Pectin gelation occurs by two general mechanisms, depending on DE. HMP requires low pH (< 3.5) and the addition of a water soluble solute, typically sucrose, for gelation, involving hydrogen bonds and hydrophobic interaction. The affinity of HMP for Ca^{2+} is generally not high enough to lead to sufficient chain association for gelation. LMP gel through the ionic interactions of polyvalent cations, such as calcium, with free carboxyl groups in the pectin backbone without sucrose (Gilsenan and others 2000). It is a notably similar manner to the stable, dimeric "egg-box" structure proposed for alginates, in which the participating sequences adopt a two-fold zigzag conformation, with chelation of calcium ions to carboxyl groups along the inner faces of both chains (Grant and others, 1973; Morris and others, 1982).

Normally, the interaction of Ca^{2+} increases with decreasing DE, and it is generally agreed that a transition in calcium affinity towards randomly charged pectins occurs around

a 40% DE (Ralet and others 2001b). Besides the methoxyl content of pectins, other intrinsic and extrinsic parameters, such as the charge distribution along the backbone, the number and size of side chains, the average molecular weight, the ionic strength, the pH, the temperature, and the presence of cosolutes, have an effect on the strength of calcium binding (Garnier and others, 1993; Voragen and others, 1995). Namely, LMP with similar ester contents prepared by different de-esterification procedures have different gelling properties due to the different distributions of free carboxyl groups along the polygalacturonic acid chains (Heri and others, 1961; Kohn and others, 1968). Willats and others (2001) studied the properties of calcium mediated gels formed from pectins containing homogalacturonan domains with differing degrees and patterns of methyl-ester group distribution. They found that the degree and pattern of methyl-esterification affects the elasticity of the gels as well as their response to compressive strain. For two pectin gels with the same distribution pattern but different DE values, lower DE pectin formed a strong gel, and 12% higher DE pectin formed a 10-fold weaker gel. In the other case, with samples having similar DE values but different distribution patterns, there was nearly a 3-fold difference in the yield point of gels formed from plant and fungal PME modified pectin. With respect to the elasticity of gels, decreasing DE was broadly correlated with increasing elasticity, as would be expected if free carboxyl groups are required for calcium cross-linking to occur. Powell and others (1982) reported that in the enzymically de-esterificated pectins (blockwise distribution) increased the Ca^{2+} binding linearly with the fraction of free carboxyl groups, whereas the chemically de-esterified samples (random distribution) showed a non-linear relationship. Other studies (Oosterveld and others, 2000;

Failds and Williamson, 1990) have reported that a partially purified pectin acetyl esterase, which also had PME, led to an improved gel formation with calcium ion.

Calcium Sensitivity

Calcium sensitive pectin (CSP) are HMP that can gel in the presence of calcium without the addition of sucrose since blocks of deesterified ester groups are present (Joye and Luzio, 2000; Hotchkiss and others, 2002). The calcium sensitivity of HMP occurs in the presence of non-homogeneous methyl ester distribution. Regions of free galacturonic acid groups form multiple calcium bridges, which create a domain of strong intermolecular associations between the galacturonan chains, resulting in increased viscosity. Consequently, extensive pectin de-methylation also causes pectin gelling or precipitation with calcium, if multiple regions of predominant free galacturonic acid groups are present on the same pectin (Ralet and others, 2003). In addition, CSP retains more water than LMP, making them softer and more commercially desirable for dietetic foods and many other acidic food applications, as well as for stabilization of casein in acidic milk-based beverage products (Swaisgood, 1997). In addition, pectins extracted from citrus pulp, which have a high DE but also contain blocks of contiguous unesterified residues, can gel through addition of Ca^{2+} (MacDougall and others, 1996; Tuerena and others, 1982).

PME can make CSP. In a study of calcium pectate gel (Powell and other, 1982), different levels and patterns of esterification by enzymic (blockwise) and chemical (random) de-esterification yielded different Ca^{2+} binding mechanisms upon gelation. In enzyme series, the extent of Ca^{2+} binding increased in an almost linear relationship with the fraction of free carboxyl groups, whereas the chemical de-esterification showed a non-

linear relationship of a form consistent with the requirement of this binding for blocks of contiguous non-esterified residues. Namely, the large free carboxyl group block size was consistent with the egg-box model for low methoxyl pectin gelation (Tuerena and others, 1982). Larger blocks were found in the enzyme de-esterified pectin than in the alkali and acid de-esterified material. Ralet and others (2003) reported that the blocks in sugar beet pectin formed by plant PME treatment allowed the formation of calcium-pectinate precipitates in concentrated mediums, even though these blocks were not long enough to induce abnormal polyelectrolyte behaviour or to promote dimerisation of pectic molecules in dilute solution. Cameron and others (2003) reported a 6.5% DE decrease of a non-calcium-sensitive citrus pectin by a salt-dependent PME from orange fruit rag tissue that increased the calcium sensitivity with no effect on molecular weight. Other studies (Oosterveld and others, 2000; Failds and Williamson, 1990) reported that a partially purified pectin acetyl esterase, which also had PME, led to improved gel formation with Ca^{2+}. Liner and Thibault (1992) suggested that a minimal block size of nine de-esterified residues was necessary for calcium cross-linking, and hypothesized that a larger de-esterified block might be necessary for gel formation.

Calcium sensitivity has been studied by physicochemical methods such as equilibrium dialysis and calcium activity determinations, mainly as a function of the degree of polymerization of the pectin and the methyl group content (Thibault and Rinaudo, 1986; Hotchkiss and others, 2002; Joye and Luzio, 2000; Schmelter and others 2002). Those studies have been carried out on dilute solutions by the determination of calcium activity

coefficients and on calcium gels, mainly by measurements of the gel strengths (Powell and others, 1982).

Rheology of Pectin Dispersion

Several models have been used to describe the flow behavior of hydrocolloid solutions, for example, linear (Newtonian or Bingham), power law (Ostwald-de-Waele), and power law with a yield stress (Hershel-Bulkley) (Marcotte and others 2001). The power law model ($\sigma = K \cdot \gamma^n$; where σ = viscosity, γ = shear rate, n = flow behavior, and k = consistency index) has been used to fit stress versus shear rate data. Depending on the range of n, these are classified in several categories based on the shear dependency: $0 < n < 1$ (shear-thinning materials), $n = 1$ (Newtonian behavior), $n > 1$ (shear-thickening materials). Shear-thinning behavior is very common in fruit and vegetable products (Steffe, 1996). Pectin dispersions exhibit Newtonia or non Newtonian depending on concentration or temperature. Marcotte and others (2001) reported that pectin dispersions exhibited a power-law shearing thinning behavior, characterized by an n value less than 1 at a concentration range of 1% to 5% and temperature range of 20°C to 60°C. In contrast, Chou and Kokini (1987) reported that hot break tomato paste pectin solutions at concentrations of 2% or lower and citrus pectin solutions at concentrations of 3% or lower were Newtonian. Marcotte and others (2001) also suggested that hydrocolloid solutions were closer to Newtonian flow at higher temperatures.

There are several factors that influence change in viscosity, like molecular weight (Mw), conformation of molecules, degree of esterification, electrolyte concentration, pH, temperature, and pectin sources (Li and Chang 1997; Phatak and others 1988; Morris and

others 2002; Marcotte and others 2001). Hotchkiss and others (2002) reported PME treated pectin decreased %DE from 70 to 32 and had a 16% reduction in intrinsic viscosity (IV) with no reduction in Mw using high-performance size exclusion chromatography with on-line multiangle laser light scattering. However, alkali deesterification rapidly reduced both Mw and IV to less than half that observed for untreated pectin. Thibault and Rinaudo (1985) showed no decrease in IV values of pectin with DE about 10, 30, and 40% after enzyme deesterification by determinating the calcium activity coefficients. Schmelter and others 2002 found that alterations of the side chain by enzymatically modification yielded pectin having a significantly lower viscosity in the absence of calcium.

Small amplitude dynamic rheological tests have been used to study the structure/network development of many food gels (Doublier and others, 1992). In a dynamic rheological test, the energy stored (G', Pa) and the energy lost (G", Pa) by samples are determined during a sinusoidal strain cycle so that both the elastic and the viscous properties are measured (Grosso and Rao, 1998; Norziah and others, 2001). For weak gel, plots of ln G' and ln G" against ln frequency (ω) have positive slopes, while for strong gels, the slopes are nearly zero. The gelling temperature of 1% pectin dispersions was approximately 15°C, since values of G" were greater than those of G' at 20°C, while at 10°C, values of G' were greater than those of G" (Rao and others, 1993). Schmelter and others (2002) found that the G' values of de-methoxylated pectin increased 35- fold and the gel-like properties were markedly enhanced in the presence of calcium. Lopes da Silva and others (1993) reported that LMP and HMP dispersions showed a quite different behavior of dynamic rheological properties caused by the higher charge density in LMP which was

related to a lower intermolecular association and a higher hydrodynamic volume. Dispersions of pectin alone or in combination with sucrose exhibited a more liquid-like behaviour with G" > G'. However, in the presence of calcium, mechanical spectra of G'> G" were obtained. Norziah and others (2001) found that increasing pectin, sucrose, and calcium concentrations increased G' and G", with pectin having the greatest effect at pH 3.0.

Zeta (ζ)- Potential of Pectin Dispersion

The surface potential can be difficult to determine. A good practical approximation is to use the ζ-potentials as an alternative. This is defined as the effective electric potential at a point in the vicinity of the shear plane of a moving particle. It can be estimated from the electrophoretic mobility of the particle, and is commonly used as an index of the electrical potential of the double layer when there are no adsorbed polymers. The ζ-potential is a useful particle characterization parameter, and is used to assess the stability of colloidal systems (Anema and Klostermeyer, 1996; Farinato and Dubin, 1999). Several electrokinetic methods are available, and all require a model of the electrical double layer in order to calculate the ζ-potential from the measured quantities. These methods include electrophoresis (particle motion in a direct current electric field), electrophoretic light scattering (Doppler shifting of scattered light due to particle motion in a direct current electric field), streaming potential, electroosmosis, diffusiophoresis, and sedimentation potential (Hidalgo-Alvarez and others, 1996; Farinato and Dubin, 1999).

There have been limited studies that reported ζ-potential of pectin solution. Nakamura and others (2003) reported the ζ-potential of soybean soluble polysaccharide (SSPS) which has a pectin like structure, pectin and their digestion products by various enzymes at pH 2~7. Pectin is an acidic polysaccharide, having galacturonic acid as a component sugar, the negative ζ- potential increases in pH. The negative ζ-potential of SSPS was smaller than that of pectin. Enzyme treatments increased the negative ζ-potential because the galacturonic acids which were not methylesterified were digested and lost from main backbone. Kulmyrzaev and others (2000) reported that some minerals bind to oppositely charged groups on the surface of emulsion droplets, decreasing the magnitude of ζ -potential and reducing the electrostatic repulsion between droplets. The ζ-potential of gum arabic stabilized oil in water emulsions has been investigated in different concentrations of NaCl over a pH range from 1 to 10 (Jayme and other, 1999). The trends observed showed decrease in negative ζ-potential with increasing salt concentration since an increase in salt concentration will lead to a compression of the electrical double layer and a corresponding reduction on ζ-potential.

Milk and Milk Protein

Milk and dairy products have played a major role in human nutrition as protein, calcium, phosphorous, magnesium, vitamin A, riboflavin and vitamin B_{12}. Additionally, it is the best characterized protein of all food proteins, both physiochemically and microscopically. Normal bovine milk contains 85 to 88% water, 3.0 to 4.0 % protein, 4.6 to 5.2% lactose, 3.0 to 5.0% milk fat, and 1.0% minerals (Walsh and others, 2000). In

nature, milk is in the liquid form. It can be described as three different ways such as an oil-in-water emulsion with the fat globules dispersed in the continuous serum phase, a colloid suspension of casein micelles, globular proteins, and lipoprotein particles, or a solution of lactose, soluble proteins, minerals, vitamins, and other components. Most constituents of milk are not present as individual molecules in solution. Instead, they exist in large, complex, associated structures. This is especially true for the caseins, which are present as large spherical micelles, and the lipids, which form even larger spherical globules (Swaisgood, 1997; Aguilera and Stanley, 1999). Milk protein is formed of the six major gene products of the mammary gland, specifically, αS_1-, αS_2-, β- , κ- caseins, β-lactoglobulins, and α-lactalbumins. Each of these proteins exhibits genetic polymorphism. Usually, milk proteins have been divided into two categories (caseins and whey proteins), depending on their solubility at 20°C when milk is acidified to pH 4.6. Both casein and whey proteins have high biological value, and contain all of the amino acids required by humans (Swaisgood, 1996; Walsh and others, 2000).

Casein and Casein Fractions

Casein is the main structure-building component of milk, especially milk gels, and is widely used in the food industry as a highly functional ingredient (Hill, 1996). There are different kinds of experiments to investigate the casein structure and to characterize the casein micelle. However, the molecular characteristics, particular tertiary structure, of the caseins remain unknown since these proteins are not typical and, apparently, cannot be crystallized (Swaisgood, 1997). Casein consists of four primary proteins: αS_1 , αS_2, β, and κ. These four primary caseins exhibit 'microheterogeneity' due to variations in the degree

of phosphorylation or glycosylation, disulphide-linked polymerization and so on (Holt,1975; Schmidt, 1982). The experimental values for the isoelectric point are pH 4.9 for αS_1-casien, pH 5.3 for αS_2-casien, pH 5.0 and 5.4 for β-casein, and pH 5.6 and 5.9 for κ-casein (Swaisgood, 1997). Due to differences in their isoelectric points, net charges are also different at other pH values. The caseins have a particular amphiphilic nature arising from a separation between hydrophobic clusters and negatively charged regions along the peptide chain, which explains the difference in their association properties. The primary sequence indicates that the hydrophobic and polar, or charged, residues are not uniformly distributed (Swaisgood, 1997; Marchesseau and others, 2002). All the caseins are relatively small phosphoproteins (19 to 25 kDa), and strongly hydrophobic in the rank of β > κ > αS_1 > αS_2 –casein (Fox, 1989). The molecular weight reported by Swaisgood (1997) was αs_1 = 22,077 ~ 23,734 Da, αs_2 = 25,157 ~ 25,400 Da, β-casein = 23,946 ~ 24,097 Da, and κ-casein = 19,800 Da.

The $\alpha S_{1,2}$-Caseins are sensitive to calcium due to the presence of phosphate groups, and precipitate in the presence of calcium ions at pH values of 7.0. The polypeptide chains contain 8.5% proline, which restricts α-helix formation. The β-Casein is the most hydrophobic casein, and forms aggregates with the N-terminal hydrophilic parts exposed to solvent and hydrophobic parts in the interior (Varnam and Sutherland, 1994). The κ-Casein differs from other caseins in that it remains soluble over a broad range of calcium ion concentrations due to the presence of only one phosphoserine group and a charged oligosaccharide moiety. It also has a relatively stable, single disulphide bonded structure,

within which are both α-helical and β-sheet region. (Walstra, 1979; Anema and Klostermeyer; 1996). κ-Casein is usually found on the exterior of the casein submicelle, and is the key to micelle structure through stabilizing the calcium-insoluble αS_1 and β-casein (Wangh and Von Hippel, 1996). The κ-Casein is thought to coat the hydrophobic core of the submicelle, and is important in casein/polysaccharide interactions due to the presence of a positively charged region available for electrostatic bonding (Langerendorf and other, 1999). It has recently been reported that the κ-casein molecules do not homogenously cover the surface due to some degree of disulfide-linked polymeric forms (Dalgleish, 1998).

Casein Micelle

Caseins are not present in milk as individual molecular structures, but rather as large colloidal micelles in association with calcium phosphate in milk at pH 6.8 (Holt and Horne, 1996; Marchesseau and others, 2002). The micelles are highly hydrated, have a voluminosity, and their size is in the range of 50 to 300 nm. Casein micelles consist of four primary proteins, αS_1, αS_2, β, and κ, in the approximate ratio 40: 10 : 35 : 12. Micelles also contain inorganic calcium, phosphate, citrate and small amounts of magnesium, sodium, and potassium. Each micelle contains 20,000 to 150,000 casein molecules, giving an average molecular weight of 2.4×10^8 (Holt,1975; Schmidt, 1982; Swaisgood, 1997). The main milk proteins are thought to be associated with the micelles in such a way that the hydrophilic negatively-charged macropeptide regions projects into the solvent as flexible hairs due to their electrostatic and hydrophobic nature (Walstra, 1979; Anema and

Klostermeyer; 1996; Rollema, 1997). Because of its amorphous character, the detailed structure of the casein micelle is not specifically known. However, the accepted view is that the particles are sterically stabilized by an external layer of κ-casein. One part of the κ-casein acts as an anchoring block (hydrophobic part), whereas the remaining charged hydrophilic part provides steric stabilization (Holt and Horne, 1996). That is, regardless of the detailed internal structure of the casein micelle, it can be observed as two interconnected domains, one inner domain comprised primarily of αS_1, αS_2, and β-caseins, which are coated and stabilized by an external domain constituted by "hairs" of κ-casein molecules (Ausar and others, 2001; Holt and Horne, 1996; Walstra, 1990). A surface layer of this type would provide stabilization through electrostatic repulsion between the charged casein micelles, as well as steric stabilization through entropic repulsions at close range due to the presence of the macropeptide hairs (Walstra, 1990).

Essentially all of the caseins are in micelles, which can be isolated by centrifugation yielding a micelle pellet, or by SEC. Other classical methods of fractionating milk proteins are by isoelectric precipitation of caseins at pH 4.6 and 20°C, or by limited proteolysis of micellar κ-casein with chymosin, resulting in coagulation of the altered casein micelles and separation of whey protein fractions (Hill, 1996; Swaisgood, 1997).

Protein and Polysaccharide Interaction

Proteins and polysaccharides are largely responsible for the structural, mechanical, and physicochemical properties of food stuffs. Thus, investigations of their interactions are primarily focused on providing optimum structural quality to the design of new and attractive foods (Beaulieu and others, 2001).

When solutions of proteins and polysaccharides are mixed, three equilibrium situations result (Williams and Phillips, 2000). Thermodynamic incompatibility due to a net repulsion between biopolymers, results in a two-phase system where each phase is enriched by one of the biopolymer species. Complex coacervation is observed for mixtures of oppositely charged biopolymers. For example, in the pH range below the isoelectric point (pI) of the protein and above the pK of the anionic polysaccharide. Usually one phase is enriched in both polymers. Miscibility is the stable dispersion of two polymers in a single phase and uncommon for mixed biopolymer solutions except in dilute systems. The conditions for complex formation or thermodynamic incompatibility are determined primarily by pH, ionic strength, charge density, and biopolymer concentration in a system (Tolstoguzov,1990). At a given mixture of protein and nonionic hydrocolloids, ionic strength and pH only influence protein self-association (repulsion between protein molecules), which is strongest at the pI due to attractive fluctuating dipole interactions, and decreases towards acidic or alkaline pH because of the increasing protein net charge (Kirkwood and Shmaker, 1952). In contrast to nonionic hydrocolloids, complex coacervation is observed for $pKa_{hydrocolloid}$ (usually around or below 3) < pH < $pI_{protein}$, where the two biopolymers carry opposite charges. At pH > $pI_{protein}$, both protein-hydrocolloid cross-association, which is less positively charged, 'anchor' points for the anionic hydrocolloid, and protein self-association, which is more protein repulsion, are reduced (Tolstoguzov, 1996).

Incompatibility, miscibility, and coacervation in dispersions virtually switch into non-adsorption, weak adsorption, and strong adsorption. Dickinson (1998) described three

kinds of structural states for a binary system of spherical particles and adsorbing polymer molecules at different polymer/particle ratios: bridging flocculation at low polymer concentrations, steric stabilization of the particles by the saturated polymer layer, and immobilization of polymer covered particles in a polymer gel network. Both adsorbing and non-adsorbing polymers are able to enhance or decrease the stability of colloidal dispersions. Adsorbing polymers can cause bridging flocculation or polymer stabilization. Bridging flocculation is restricted to low concentrations of polymers with multiple anchor sites, and a spatial extension beyond the range of the repulsive barrier between colloid particles. Under these conditions, the surfaces of the colloidal particles easily become linked by polymer bridges, at distances that prevent any impact of the repulsive barrier. Efficient polymeric stabilization is achieved by the combination of high molecular weight, high surface coverage, and a blockwise distribution of anchor points (Dickinson and Stainsby, 1982; Syrbe and others, 1998). Non-adsorbing, free polymer at moderate concentrations usually leads to destabilization of the colloidal dispersion. Flocculation or separation into two phases, one concentrated and the other depleted in colloidal particles, is possible (Sperry and others, 1981; Fleer and others, 1993).

Milk Protein and Pectin Interaction

Interactions of casein with pectin are widely employed in the control of texture and stability of certain dairy products such as fruit milk drinks, yogurt drinks, soymilk, and whey drinks. Because of the polyelectrolyte character of milk proteins and pectin, electrostatic interactions play an important role in determining mixed biopolymer behavior (Dickinson, 1998).

Mishra and others (2001) reported that whey protein and pectin increased solubility and emulsifying properties at pH 4.6 as compared to the control whey protein without pectin. Some studies made a comparison between HMP and LMP. In acidified casein dispersion, Peryra and others (1997) found that HMP increased the stability, due to anchoring of HMP to the casein surface as well as interaction of HMP with solvent. In contrast, LMP-casein dispersions displayed greater intermolecular interactions. Girard and others (2002) also investigated the types of interactions involved in β-lactoglobulin (lg)/LMP and β-lg/HMP systems. They found that the amount of β-lg that formed complexes was greater with LMP than with HMP. Namely, 96% of β-lg complexed with LMP, whereas only 78% of β-lg complexed with HMP at pH 4.5. In the other hands, Dickinson and others (1998) studied stability of emulsions with the same pectin content that were made with αS_1 -casein, β-casein, or sodium caseinate. Those three emulsions showed a similar average droplet size and stability properties, except in the case of αS_1-casein based emulsions at ionic strength > 0.1 M. For αS1-casein, it is not influenced by pectin addition because it has only extensive flocculation.

Some studies reported the difference between pectin and other polysaccharides. Leroux and others (2003) reported that pectin produced fine and stable emulsions similar to those of gum arabic which is the most common emulsifier, but at much lower dosages, because pectin may take up a greater volume around the droplets. This could be due to the more extended conformation of the pectin molecules. Paraskevopoulou and others (2003) evaluated the ability of three polysaccharides (xanthan, guar gum, HMP) to prevent casein coagulation and sedimentation in an acid environment. Xanthan appeared to be more

effective at relatively low concentration levels (0.2%) as compared to guar gum, while HMP was less effective, even at concentrations as high as 1%. Particle size and rheology measurements indicated weak gel properties of xanthan solution in stabilizing the system against "wheying off". Other interactions, such as particle network formation as a result of depletion flocculation, occurred with guar gum or pectin adsorption at the casein particle surface and resulted in less steric stabilization than xanthan.

Application of Pectin in Acidified Milk System

Milk processors have been concerned with the stability of colloidal casein-calcium-phosphate phase of milk. Destruction of this stability by acid or by the use of an enzyme is an essential step in the preparation of cheese, and the texture and quality of the product is often dependent upon this step. Conversely, the maintenance or recovery of colloid stability is essential for all milk beverage products (Rose, 1965).

Stabilizers, also known as hydrocolloids or gums, are a class of food-grade water-soluble polysaccharides of high molecular weight. Traditionally, they have been regarded as thickening agents, whose functionality relies on viscosity enhancement, gel formation and water-binding ability (Hansen, 1982). Recently hydrocolloids have used in dairy products to improve product texture and to increase the shelf-life of dairy products. Otherwise dairy products suffer from wheying off, settling of dispersed particles, or creaming and flocculation of emulsion droplets (Syrbe and others 1998).

Acid milk drinks, like fruit milk drinks, yogurt drinks, soymilk, butter-milk, whey drinks, kefir, and others, can be described as an acidified protein liquid system with stability and viscosity similar to natural milk. This liquid system is usually composed of an

acid dairy phase (fermented base) or a neutral base (milk, soy milk) with an acidic medium (fruit phase), sugar, and stabilizer (Laurent and Boulenguer, 2003).

Pectins are employed as a means of achieving stability in low pH dairy-based products. As pH decreases, milk protein gains further charge, and electrostatic repulsion is changed. Thus, acidifying casein micelles leads to instability. At this point, by the addition of a sufficient amount of pectin, the milk system remains a stable liquid upon acidification (Tuiner and others, 2002). Pectin stabilization of milk proteins under acidic conditions, below pI of milk protein, is caused by electrostatic repulsion. Namely, pectin is adsorbed onto the surface of casein micelles by electrostatic attraction, and the negatively charged pectin-casein complex is then dispersed by electrostatic repulsion.

HMP, with a degree of methyl esterification in galacturonic acid of more than 50%, in particular, has generally been used as a stabilizer of acidic beverages to prevent the formation of sediment (Fleer and other, 1984; Maroziene and De Kruif, 2000). Laurent and Boulenguer (2003) found that calcium sensitive pectin is more efficient than the non-calcium sensitive pectin in terms of stabilization in acid dairy drinks (ADD). However, the non-calcium sensitive pectin is also quite good for stabilizing ADD, demonstrating that calcium sensitivity is not the only parameter governing stability. Moreover, plant PME modification of pectin leads to improved protein stabilization, characteristic for high ester pectins (Christensen and others, 1997). Nakamura and others (2003) have reported that soybean soluble polysaccharides (SSPS) which have a pectin-like structure could be used as dispersion stabilizers for milk protein under acidic conditions in a manner similar to HMP. In this case, the neutral sugar side chain of SSPS plays a significant role as a stabilizer.

Analysis of Dairy and Pectin Mixtures

Particle Size of Casein

Micelle sizes are the range from 50 to 300 nm, and each micelle contains 20,000 to 150,000 casein molecules, giving an average molecular weight of 2.4×10^8 (Schmidt, 1982). In skim milk from eight cows, Holt (1975) reported that the weight average molecular weight of the particles varied from $2.6 \cdot 10^8$ to $15 \cdot 10^8$ and the corresponding average particle radius varies between 90 and 130 nm. These various particle sizes reveal the particle size heterogeneity in skim milk dispersions. Schmidt and others (1973) reported particles with a diameter of less than 20 nm, which correspond in size to casein submicelles, accounting for nearly 80% of the number of all casein particles. In addition, depending on pH, casein micelles aggregated various. Saito (1973) reported different precipitates in the pH range 2 to 6. Namely, casein micelles aggregated in the pH range 3 to 5, and the precipitates were extremely fine at pH 4-5.

Several studies make a comparison among individual casein fractions. Schmidt and Buchhem (1975) reported that the size distributions in solutions of αS_1- casein differed considerably from those of β-, and κ-casein fractions. The αS1- casein showed a steady decrease in particle numbers with increasing diameter, whereas β- and κ-casein exhibited a maximum in their particle size distributions. This difference may be explained on the basis of the known association behavior of the individual casein components. αS1- Casein undergoes a series of consecutive association steps, of which the equilibrium constants are of the same order of magnitude (Schmidt, 1970). This results in a broad distribution of monomers and polymers, in which the number of polymer molecules decrease with the

degree of polymerization and, thus, with their size. In contrast to the indefinite association of αS_1- casein, β-casein shows a discrete, micellar type of association (Schmidt and Payens, 1972). Rose (1965) reported that β-casein content decreased with decreased micelle size. Generally larger particle sizes result in more unstable dispersions, which are prone to syneresis and "wheying off" (Glahn, 1982).

Several methods to measure the size of milk and casein dispersions. Electron microscopy has been used extensively to investigate the size of casein micelles in raw and processed milk (Calapal, 1968; Schmidt and Buchheim, 1975; Mangino and Freeman, 1980). A combination of light scattering and centrifugal fractionation was used by Schmidt and Payens (1972) for the determination of micelle size. Normally, the average particle sizes determined by electron microscopy were smaller than those found by light scattering due to shrinkage of the micelles on the electron microscope grid (Holt, 1975). Pepper (1972) used gel chromatography for measuring a hydrodynamic diameter in casein micelle.

Zeta (ζ)-potential of Dairy and Pectin Mixtures

The stability of the casein micelles has been attributed in part to the net negative charge on the surface of the micelles. The surface charge of colloids is often estimated by ζ-potential, which can be derived from the electrophoretic mobility of the particle. The ζ -potential is a measure of the magnitude of the repulsion or attraction between particles, and its measurement brings detailed insight into the dispersion mechanism, and is the key to electrostatic dispersion control (Anema and Klostermeyer, 1996; Farinato and Dubin, 1999).

At neutral pH, ζ-potential for casein micelles were reported around –18 mV~ -20 mV because carboxyl groups partly became negatively charged ($-COO^-$) and partly because neutral ($-NH_2$). Between pH 5.8 and 5.5, ζ-potential of milk decreased and conduced the micelles to form clusters which was particle size change from 180 to 1300 nm (Dalgleish, 1984; Laurent and Boulenguer, 2003; Theresa and others, 1996). Below pI_{pro} (~ pH 5.0), milk dispersions showed positive ζ-potential because the amino groups are positively charged ($-NH_3^+$), whereas the carboxyl groups are neutral (-COOH). In contrast to casein dispersions in the absence of pectin, adding pectin makes the dispersion contain a more negative charge on the surface of the colloids. Under acidic conditions, below the isoelectric point of milk protein, pectin added milk protein increase negative charge or decrease positive charge on the casein dispersions because pectin is acidic polysaccharides having galacturonic acid as a component sugar (Nakamura and others, 2003). Therefore, at that point, pectin is adsorbed onto the surface of casein micelles by electrostatic attraction, then the negatively charged pectin-casein complex is dispersed by electrostatic repulsion (Fleer and other, 1984; Maroziene and De Kruif, 2000). Moreover, those charged complexes of casein fractions and pectins could be advantageously used in combination with other charged components (biopolymers, surfactants, lipids) to obtain new functional supramolecular entities (Schmitt and others, 1999).

Viscosity of Dairy and Pectin Mixtures

In skim milk, casein micelles are the main contributors to the viscosity of milk. Normal milk behaves as a Newtonian liquid, and its viscosity is affected by temperature, fat content, protein content, total solids, and solid-to-liquid fat ratio (Randhahn,1973; Walstra

and Jenness, 1984; Langley and Temple, 1985; Velez-Ruitz and Barbosa-Canovas, 2000). Moreover, there is a transition from Newtonian to non-Newtonian behavior in the skim milk system as the concentration is increased. It is due to the removal of water which causes an increase in volume fraction of dispersed particles and which increases the micelle-micelle interactions as the distance between the micelles becomes smaller (Walstra and Jenness, 1984). For example, the dispersions of LMP-casein or HMP-casein were less Newtonian and displayed higher consistencies than LMP or HMP (Pereyra and other, 1997).

The viscosity of the dispersion influences the adsorption and bridging of the pectin onto/between the micelles (Walstra and Jenness, 1984; Maroziene and De Kruif, 2000). In pectin-containing acidified milks, Glahn (1982) reported that viscosity had an initial sharp increase at lower pectin levels and followed by a sharp decline. Maroziene and De Kruif (2000) reported that the addition of pectin to acidified skim milk increased the viscosity only minimally. Laurent and Boulenguer (2003) compared calcium sensitive (CS) and non-calcium sensitive (NCS) as stabilizers in acid dairy drinks. For CS pectin, the viscosity observed at high pectin concentrations was much higher and it was explained by the presence of a network and depletion phenomena for the CS pectin. Some studies reported the viscosity of casein containing pectin based on individual casein fractions and these results demonstrate the difference in protein-polysaccharide interactions for individual casein monomers, which has been previously recognized in the case of casein with κ-carrageenan (Snoeren and others, 1975; Skura and Nakai, 1981; Ozawa and others,1984; Dalgleish and Morris, 1988). Dickinson and others (1998) demonstrated the significantly higher viscosity of the HMP-containing αS_1-casein stabilized emulsion at pH > pI_{pro}. It can

be attributed to attractive protein –polysaccharide interactions at the surface of the emulsion droplets that are absent from the corresponding β-casein system.

Microstructure of Dairy and Pectin Mixtures

Milk products are rich in microstructural elements, such as plastic globules, membranes, colloidal aggregates, and even crystals, and all these elements interact to form the variety of textures characteristic of dairy products (Aguilera and Stanley, 1999). Various microscopy techniques have been extensively used to investigate the ultrastructure of milk components, the changes they undergo, and the interactions that occur among them as well as between them and other ingredients during processing (Saito, 1973). Snoeren and others (1975) demonstrated by electron microscopy that κ-casein and κ-carrageenan molecules in solution showed fibre-like structure, and that multi-stranded structures were absent in solutions of κ-carrageenan. In addition, microscopy techniques have also been used extensively to investigate the size of casein micelles in raw and processed milk (Calapal, 1968; Scdhmidt and Buchheim, 1975; Mangino and Freeman, 1980). Schmidt and others (1973) found particles with a diameter of less than 20 nm, which correspond in size to casein submicelles, to account for nearly 80% of the number of all casein particles present.

Normally, the microstructure of milk or milk and pectin mixtures has been studied in mixed gel systems, and only a few studies have reported microstructure in liquid systems (Willson and other, 1961; Mangino and Freeman, 1980) without any further preparation of the samples. For distinguishing the junction of mixture or individual component, samples were stained. As in previous studies, Fast Green has been used for staining the whey

protein (Dumay and others, 1999; Beaulieu and others, 2001). Immunolocalization studies using monoclonal antibodies, such as PAM 1 and PAM 2 that bind to un-esterified and un-substituted pectin, or JIM 5 and JIM 7 that bind to pectin depending on a range of a degree of methyl-esterification, have been important in allowing for observation of the distribution of pectin (Willats and others, 1999; Willats and others, 2001).

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CHAPTER 3

ACTION PATTERN OF VALENCIA ORANGE PME DE-ESTERIFICATION OF HIGH METHOXYL PECTIN AND CHARACTERIZATION OF MODIFIED PECTINS[1]

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Abstract

Two Valencia PME fractions, B-PME containing 36 kDa and 13kDa peptides, and U-PME, containing a 36 kDa and 27 kDa peptides, were used to de-esterify pectin from 73% degree of esterification (%DE) to 63% and 61%DE, respectively. The chemical structures of original (O-Pec), B-PME modified pectin (B-Pec), or U-PME modified pectin (U-Pec) was evaluated for % DE, molecular weight, charge distribution, and ζ-potential. The main component of O-Pec eluted from ion exchange chromatography in a relatively narrow peak at low salt concentration and a smaller component eluted at higher ionic strength. B-Pec and U-Pec eluted as one broad peak near the same ionic strength as the second, smaller fraction of O-Pec. PME modification did not change molecular weight: O-pectin (134,000 g/mol), U-Pec (133,850 g/mol) and B-Pec (132,250 g/mol). The NMR signal of GG and GGG increased after modification, and the signal of EE and EEE decreased. The negative ζ-potential increased with increase in pH for all pectins. U-PME and B-PME created differently modified pectins that vary in degree and length of multiple attacks and fraction of the pectin population that was modified.

Key words: PME, Modified pectin, charge and charge distribution, NMR, IEX, molecular weight, ζ-potential

Introduction

Pectins are a complex group of structural polysaccharides with an important role as primary constituents in the cell walls of plants and also as gelling agent in food systems. Pectins are anionic polysaccharides consisting of a linear backbone of α (1-4)-D-galacturonic acid partially esterified with methoxy ester. The homogalacuturon backbone may be interrupted with 1, 2 linked L-rhamnose residues with other neutral sugars attached as side chains (De Silva and others 1995).

Pectin methylesterase (PME, E.C. 3.1.1.11) catalyses the demethoxylation of pectins. It has been isolated from various sources and has different action patterns with respect to the removal of methoxyl esters. Acidic microbial (*Aspergillus japonicus, Aspergillus niger, Aspergillus foetidus*) PMEs de-esterify pectins to form a random distribution of free carboxyl groups (Ralet and others 2001b; Thibault and Rinaudo 1985). The action of alkaline PMEs from higher plants (banana, tomato, orange, apple, strawberry) and from fungi (*Trichoderma reesei*) catalyze demethylation of pectin linearly along the chain (single chain mechanism) and result in blockwise arrangement of free carboxyl groups. This gives rise to block-structures, adjacent free galacturonic acid units on the homogalacuturon backbone, which allow calcium cross-linking of pectin chains (Limberg and others, 2000; Hunter 2002; Savary and others 2002). Some plant PMEs have the capacity to remove a limited number of methyl esters per reaction, yielding short un-esterified blocks (Denes and others, 2000b; Willats and others 2001). In a study on apple PME by Denės and others (2000), the action patterns at pH 7.5 consisted of a blockwise

distribution by a single chain mechanism, while the action at pH 4.5 was also a blockwise distribution, but with shorter blocks on multiple chains.

Multiple PME isozymes are present in orange. Individual isozymes can be distinguished by their expression patterns, and by their physical and biochemical properties (Bordenave, 1996). Major PME isozymes have been isolated from Navel orange (Versteeg and others 1980) and Valencia orange (Cameron and others 1998) that differ in thermostability and ability to rapidly clarify orange juice. Wicker and others (2002) showed clarification of citrus juices by thermolabile PME from Valencia pulp occurred only in the presence of cations and suggested that cations moderated PME activity. Ackerley and Wicker (2003) also reported that rapid clarification was associated with a thermolabile PME that contained 36kDa and 27kDa peptides and PME extracts that contained 36kDa and 13kDa peptides did not rapidly clarify juices. Wicker and others (2003) described the juice clarification and pectin modification potential of Valencia PME isozymes. They suggested that functional properties of pectins may be related to differences in the extent and pattern of de-esterification. Savary and others (2002) identified three peptides with PME activity with estimated molecular weight values of 34kDa, 27kDa, and 8kDa. They also reported that the N and C terminus of the 36 kDa peptide was nearly identical to the N and C terminus of the 27 and 8 kDa, respectively. A band at 36 kDa also was reported by both Christensen and others (1998) and Nairn and others (1998). Hotchkiss and others (2002) demonstrated that salt-independent orange PME modified pectin charge which produced a calcium sensitive pectin while preserving its molecular

weight (MW). Hunter (2002) also mentioned there was no MW change in modified pectin by Valencia PME containing 36kDa and 13kDa peptides.

Based on the hypothesis that PME fractions containing the 36 and 27 kDa peptides will yield differently modified pectins than PME fractions containing the 36 and 13 kDa peptides, the objective of this study was to use two different Valencia PME fractions to de-esterify pectin to a target DE value and characterize the resultant pectin product for charge and charge distribution. Ultimately, the availability of different enzymes could enhance the structural characterization of pectins and correlation with functional properties. The information would be potentially useful in developing tailed pectins for applications such as a stabilizer in acidified dairy products.

Materials and Methods

Materials

Crude Valencia PME extract was prepared fromValencia orange pulp (donated by Citrus World, Lake Wales, FL) and commercial, unstandardized high methoxyl pectin (Citrus pectin type 104, high methoxyl, CP Kelco, Lille Skensved, Denmark) was used as pectin source.

Valencia PME Preparation and Characterization

PME extract was prepared as described by Wicker and others (1988). Crude extract was extracted from frozen pulp with 0.1 M NaCl, 0.25 M Tris buffer, pH 8 at a ratio of buffer to pulp of 4:1. The extract was homogenized (Pro 300A, Proscientific Inc., Monroe, CT) for 1 min at 4°C. After adjustment of pH to 8.0, the homogenate was filtered through

Miracloth (CalBiochem, La Jolla, CA). The filtrate was concentrated by 30% ammonium sulfate precipitation overnight at 4°C, and centrifuged (Sorvall RC-5B centrifuge, Dupont Instruments, Doraville, GA) at 8000 g, 4°C for 20 min. The supernatant was dialyzed overnight against 50 mM sodium phosphate, pH 7. The dialysis tubing (Spectra/Por, MWCO 6000, Fisher Scientific, Atlanta, GA) was boiled in 10% acetic acid and rinsed in deionized water to minimize loss of PME activity. After dialysis, the enzyme extract was filtered through Miracloth.

To prepare PMEs, chromatography was performed using an Äkta Prime system (Amersham Pharmacia Biotech, Uppsala, Sweden) following the modified method of Ackerley and Wicker (2003). All buffers were degassed and filtered through an 0.45 μm filter (Whatman, Clifton, NJ) before use in chromatography. The crude PME extract was first loaded onto a 5 mL Hi-Trap SP cation exchange column (Amersham Pharmacia Biotech, Uppsala, Sweden) at 5 mL/min. PME that did not bind Hi-Trap SP column was loaded onto a 5 ml Heparin (HP) affinity column. PMEs were eluted with 10 mM Na phosphate, pH 7 and 10 mM Na Phosphate/ 1M NaCl, pH 7 gradient. The PME activity in fractions was qualitatively identified using a pH sensitive dye to detect pectin methylester hydrolysis (Corredig and others 2000). PME active fractions were quantified and pooled. Bound PME that eluted from the Heparin column was denoted bound PME (B-PME). PME that did not bind the Hi-Trap SP, nor the heparin column were denoted unbound PME (U-PME).

The PME activity of the purified Valencia PME was determined by a pH stat titrator (Brinkmann, Westbury, NY) at 30°C in 1% high methoxyl pectin (Citrus pectin type CC104, Citrus Colloid Ltd., Hereford, U.K.) and 0.1 M NaCl at a set point pH of 7.5. A unit of PME activity was defined as the microequivalent of ester hydrolyzed/min at 30°C.

The amount of protein was quantified by Bradford protein assay (Bradford, 1976) using a Microplate Reader (MPR Model 550, Bio-Rad Inc., Hercules, CA). Protein subunit composition of fractions was performed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Amersham Pharmacia Co., Uppsala, Sweden). Samples were diluted to a constant protein concentration and a 5 µl aliquot was run on a PhastGel gradient gel, 8-25%. The gel was stained with silver stain according to manufacturer specifications (Amersham Pharmacia Co., Uppsala, Sweden). The staining intensity of the bands on SDS-PAGE was measured by densitometry with a scanner (Model GS-670, Bio-Rad Inc., Hercules, CA).

Pectin Modification and Fractionation

Pectin (2% w/v) was hydrated in 10 mM EDTA solution and washed with ethanol and acetone. Washed pectin was placed in glass pans and left to dry in the fume hood over night. Washed pectins were ground using an ultra centrifugal mill (ZM 100, Retsch, Haan, Germany) with 0.25 mm sieve and labeled as original pectin (O-Pec). To modify pectins, O-Pec was de-esterified by U-PME or B-PME to a targeted %DE value. A 1% pectin dispersion in 0.1N NaCl was equilibrated to 30°C and adjusted to pH 7.5 with 2N NaOH. Valencia U-PME or B- PME was added at 3.0 units/g pectin, and the pH was maintained at

pH 7.5 with 0.5N NaOH for the calculated time to achieve the target DE. The PME activity in the dispersion was stopped by the addition of 95% ethanol and boiling for 10 min. After cooling to room temperature, the modified pectin was washed with ethanol and acetone. After drying and grinding, modified pectins were labeled as U-Pec and B-Pec, respectively, according to U-PME or B-PME, respectively, used to make the modification.

To fractionate O-pec, B-Pec and U-Pec, chromatography was performed with an Äkta Prime (Amersham Pharmacia Biotech, Uppsala, Sweden) with an XK-50 column (Amersham Pharmacia Biotech, Uppsala, Sweden), 500 ml column volume, Macro-Prep High Q Support (Bio-Rad, Hercules, CA). A 2% (w/v) pectin dispersion in 0.5 M acetate buffer, pH 5.0 was filtered through two layers of Miracloth (Calbiochem, LaJolla, CA). After degassing, the pectin dispersion was gently mixed into the equilibrated anionic exchanger, and equilibrated for 1hour at room temperature. Using a flow rate of 20 mL/min, the pectin sample and packing material were packed into the column, washed with column volumes of 0.5 M acetate buffer and eluted with gradient of 0.5 to 1.3 M acetate buffer, pH 5.0 at flow rate of 12 ml/min. Based on galacturonic acid assay of fractions (Blumenkrantz and Asboe-Hansen 1973, fractions were pooled into four large fractions, dialyzed against deionized water, freeze dried (Unitop 600L, Freeze Mobile 25SL, VisTris, Gardiner, NY), and ground (ZM 100, Retsch, Haan, Germany) for the further study.

Characterization of Modified Pectin

Ion Exchange Chromatography

Analytical ion exchange chromatography (IEX) was used to estimate charge distribution. Using a flow rate of 2 mL/min, the pectin samples were loaded onto a 5 mL Q column (Bio Rad, Hercules, CA, USA), equilibrated in 0.5M acetate, pH 5.0, and then eluted through the gradient of 0.5 to 1.3 M acetate buffer, pH 5.0. After the IEX elution, uronic acid content was analyzed using the m-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen 1973).

^{1}H NMR Spectroscopy

To determine the % DE of pectins, ^{1}H NMR spectroscopy was performed using the modified method of Andersen and coworkers (1995). Pectin samples for NMR analysis were lyophilized five times with D_2O (6mg pectin in 0.7ml 50mM phosphate buffer, pH 7.0 in D_2O and four times 1ml D_2O) to remove most solvent protons. Then the samples were dissolved in 0.75ml 99.96% $2H_2O$. ^{1}H spectra were acquired with a Varian Inova 500 spectrometer (Varian, Inc., Palo Alto, CA) at 80°C using the presaturation experiment to suppress residual water signal in the sample. The concentration of single proton from 0.04% water is about 45 mM. Without water suppression, the water signal will severely overlap with the ^{1}H resonances for the measurements. The data were processed and analyzed using VNMR 6.1C software of the NMR spectrometer. The ^{1}H chemical shift is internally referenced to the water resonance of 4.26 ppm (Rosenbohm and others, 2003). The NMR spectra were measured at 80°C with dilute pectin sample with phosphate buffer made in D_2O, at pH 7.0 in order to decrease the viscosity and increase the solubility of the sample. In preliminary trials, 1M NaOD (55µl) added in the

NMR tubes before measuring NMR gave a poor spectral peak. ^{1}H NMR resonances were assigned according to the published assignments (Rosenbohm and others, 2003; Denës and others, 2000a). The values of DE and the probabilities of dyads and triads fractions are quantitatively determined from the integration volumes of the assigned spectral peaks based on the relationships (Gradsalen, and others, 1996):

$$DE = I_E(H\text{-}4) / I_E(H\text{-}4) + I_G(H\text{-}4)$$

$$F_{GGG} = I_{GGG}(H\text{-}5) / I_E(H\text{-}4) + I_G(H\text{-}4)$$

where I represents the integration volumes, E and G are denoted to esterified and de-esterified resonances, respectively. The overlapped peaks were deconvoluted using the VNMR software.

HPSEC-Multi Angle Light Scattering

Molecular weights (Mw) were determined as described by Corredig and others (1999) using an HPSEC-multi angle light scattering system consisting of a Waters P515 pump with an in-line degasser (Waters, Milford, MA) and two in-line filters (0.22 and 0.10 mm pore size, Millipore, Bedford, MA). Dispersions of pectin in 50 mM sodium nitrate (3mg/ml) were filtered through 0.8 μm (polypropylene, 25 mm, Whatman, Maidstone, England). The mobile phase was 50 mM sodium nitrate, sequentially filtered through 0.2, 0.1, and 0.1 μm filters (47 mm, Gelman Sciences, Ann Arbor, MI). Separation was achieved by using a guard column and two PL-Aquagel-OH linear mix columns (8 μm pore size, Polymer Laboratories, Inc., Amherst, MA) connected in series. A multi-angle light scattering detector and a refractive index detector were connected in series (Wyatt

Technologies, Santa Barbara, CA). The multi-angle light scattering detector (DAWN DSP-F) was equipped with a P10 flow cell and a He-Ne laser-light source (633 nm). The refractive index detector was an Optilab DSP interferometric refractometer operating at 633 nm. Data was processed using the ASTRA/Easi SEC software (vs. 4.74.03). Molecular weight as a number average (M_n), weight average (M_w) and z-average (M_z) was calculated for each sample. Data presented is the average of three replications. Specific refractive index increment (dn/dc) values were determined with the Optilab using a syringe pump (Razel Scientific, Stamford, CT). Serial dilutions were made (ranging from 0.06 to 1.2 mg/ml) to determine the slope of the increment. Results were processed using the software (vs. 5.2) provided by the manufacturer (Wyatt Technologies, Santa Barbara, CA).

Zeta (ζ)- Potential

Measurement of ζ- potential was performed by a modified procedure of Nakamura and other (2003) using 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. A 0.4% (w/w) pectin solution was adjusted to the pH range of 3.0 to 7.0 with HCl and NaOH. All experiments were carried out at 25°C with the laser beam operation at 659.0 nm and 1.330 as the refractive index. The measurements were carried out in triplicate with 3 runs of 2 min each and 5 sec between each run. The ζ - potential was determined subsequently after the particle size determination for the same sample of pectin solution. The effective diameter of the particles in solution was calculated from a

cumulative fit of the intensity autocorrelation function obtained by the intensity fluctuation of the scattered light (Dagleish and Hallett, 1995) with 90-Plus particle sizing software (version 3.37, Brookhaven Instruments, Worcestershire, UK). The measurements were carried out in triplicate with 5 runs of 2 min between each run.

Results and Discussion

PME Characterization

A description of Valencia PME fractions used for pectin modification are summarized in Table 1. There is no significant difference in protein content between the two PMEs fractions but crude PME had 5 - fold higher protein content than U-PME or B-PME ($p < 0.05$). For PME activity and specific activity, U-PME showed higher value than B-PME. The specific activity was U-PME (101.31 PEU/mg protein), crude PME (68.29 PEU/mg protein) and B-PME (25.55 PEU/mg protein), respectively.

Based on SDS-PAGE analysis (data not shown data), separation of PME after two different columns, resulted in PME fractions with different peptide bands. Crude PME extract indicated peptides at 36kDa, 27kDa, and 13kDa. B-PME had dominant bands at 36kDa and at 13kDa. U-PME had a 36kDa and 27kDa peptide. Those three bands were similar with bands from the purified commercial orange PME which has a dominant band estimated 34kDa, and secondary bands at 27kDa, and about 8kDa (Savary and others, 2002) while a band at 36kDa migrated as 34kDa. In a study by Cameron and others (2003), they found two peaks, peak 2 (33.5kDa and pI 9.2) and peak 4 (33.4kDa and pI 10.1) from two salt-dependent orange PMEs through heparin and CM-Sepharose chromatography.

IEX chromatography of PME resulted in B-PME and U- PME to obtain different enzyme activity and peptide bands.

Chemical Structural Properties of Modified Pectins and Their Pectic Fractions

Charge Distribution of Pectins by IEX

The distribution of methoxyl groups was studied by analytical IEX, using gradient elution. The elution profile of the O-Pec is polydisperse (Figure 1). The main component eluted at lower salt concentrations and a smaller fraction eluted at higher ionic strength. The main peak of B-Pec and U-Pec eluted as one broad peak near the same ionic strength as the second, smaller fraction of the O-Pec. This elution of B-Pec or U-Pec shifted to higher salt concentration suggested increased charge density. That is, de-esterification by Valencia PMEs produced B-Pec and U-Pec with a small change in the DE, but a completely different pattern towards IEX from O-Pec because of the change of charge density.

As observed by IEX (Kravtchenko and others, 1992b; Tuerena and others, 1982), pectins eluted in a relatively narrow peak. The broadness of peaks indicated that pectin charge was distributed over a wide range of DE. Schols and others (1980) reported pectins with a random and blockwise distribution of methoxyl groups elute in narrow and broad distribution curves, respectively. The broad elution of citrus PME demethylated pectins was explained by the separation based on intermolecular charge density and not the total charge of the molecules. Anger and Dongowski (1984) also showed the difference in the distribution of the free carboxyl groups along the pectin backbone in IEX elution. A

blockwise distribution might result in zones of higher charge density, which bind strongly to the ion exchanger.

Degree of Methyl Esterification

The %DE of unfractionated and fractionated pectins from ^{1}H NMR spectra is shown at Figure 2. The initial %DE of 73% was deceased to 63% in B-Pec and 61% in U-Pec, respectively. The experimental %DE was slightly less than the targeted %DE and slightly different between U-Pec and B-Pec. The difference may be likely related to summative experimental error in assay for PME, timing and terminating of modification, and NMR analysis for %DE.

Based on elution of uronic acid content into fractions, O-Pec was pooled into five fractions, but fraction 1 was lost. Tubes of U-Pec and B-Pec were pooled into four fractions. Fractionation of B-Pec and U-Pec by IEX allowed the collection of pectin populations with similar charge and charge density. Usually, high DE values and less charge dense pectins elute first and as elution volume increase, %DE decreases (Ralet and others, 2001a). Kravtchenko and others (1992a) reported that structural features other than average DE govern the strength of binding to an anion exchange column. Kravtchenko and others (1992b) reported that the DE of the fractions by IEX of three samples decreased regularly from fractions 2 to 8, but pectin fractions with a DE different from that expected had a higher phenolic content. In chromatography of O-Pec, B-Pec, and U-Pec, the %DE decreased with increase in fraction number. Moreover, the first eluting pectin fraction had higher a DE value than unfractionated pectin. Because the DE of unfractionated pectin is

an average DE values, the pectin population may have higher or lower DE value. U-Pec eluted over a more narrow range of DE values (between 69 and 49 %DE) than O-Pec (76-45 %DE) or B-Pec (65-33 %DE). This homogeneous charge and charge density of U-Pec relative to B-Pec suggests a different mechanism of action for the two modified pectins. Apparently, U-PME is able to de-esterify a large population of pectin, while B-PME is able to create greater charge density on fewer pectin molecules

Molecular Weight and Particle Size

All unfractionated and fractionated pectins are polydisperse with Mw/Mn ratios ranging from 1.30 to 2.11. For unfractionated pectin, O-Pec, B-Pec, U-Pec showed 134,000 g/mol, 132,000 g/mol, and 134,00 g/mol, respectively (Figure 3-1) and there was no significant difference after modification ($p > 0.05$). The IEX fractionation reduces polydisperse to the some extent. After IEX fractionation, Mw of fractionated pectins was significantly lower than unfractionated pectins and the yield of pectin recovered from the column was between 60 and 80%. This may be due to high viscosity of the concentrated pectin samples resulted in elution of part of the pectin at IEX by the ions present in the injected sample (Schols and others, 1980). Some highly de-esterified pectin aggregates may be irreversibly stuck on the column. Among fractionated pectins, there was no significant difference in Mw regardless of modification except for the latest eluting fractions of O-Pec and U-Pec (Figure 3-2).

In a study via SEC and light scattering, fractions of a given hydrodynamic volume within one pectin sample remained highly heterogeneous on the basis of their molecular

weight, indicating the coexistence in pectins of particles of very different shapes and DE (Kravtchenko and others, 1992a). Accurate determination of the Mw distribution is extremely difficult because of the heterogeneous nature of pectin such as the presence of smooth and hairy regions, and the varying inter-and intramolecular distribution of methyl esters (Daas and others 2001). Some studies (Hotchkiss and others, 2002; Hunter, 2002; Cameron and others, 2003) showed the preservation of Mw after orange PME de-esterification regardless of PME isozyme. Thus, it is hard to distinguish the difference between B-Pec and U-Pec by Mw or effective diameter.

^{1}H NMR analysis

^{1}H NMR spectra of unfractionated and fractionated pectins which differentiate between dyads, and triads in partly esterified galacturonic acid are shown in Table 2. There were three main signal groups, the protons H-1, H-4 and H-5 in the G and E residues of ester galacturonans. The protons were shifted slightly downfield compared with other researchers, because chemical shift of protons of E (esterified galacturonic acid) and G (de-esterified galacturonic acid) residues were dependent on the nature of their neighboring units (Denès and others, 2000).

The spectra of unfractionated pectins are depicted in Figure 4-1. Since there was a 10% DE decrease from O-Pec to U-Pec or B-Pec, the intensity of G increased and the intensity of E decreased in order of O-Pec, B-Pec and U-Pec. At the dyads and triads in partly esterified galacturonic acid, the Valencia PME modification showed different spectra among pectin samples. The signal of GG and GGG of B-Pec and U-Pec increased

compared to O-Pec. B-Pec and U-Pec showed similar frequency at GG and GGG. In contrast, the signal of EE, EGG, and EEE of B-Pec and U-Pec decreased. However, the signal associated with F_{EE}, F_{EEE}, and F_{EGG} in B-Pec and U-Pec was slightly different. B-Pec exhibited closer frequency to O-Pec than U-Pec in those signals. For the signal of GE, EG, and GGE, there was no difference among O-Pec, B-Pec, and U-Pec.

In the fractionated pectins (Figure 4-2, 4-3-, and 4-4), the signals of peaks show greater differences compared to peaks from unfractionated pectins, especially protons H-1 which represents triads GGG, EGG, GGE and EGE. Later eluting IEX fractions showed higher F_{GG}, and F_{GGG}, but lower F_{EE} and F_{EEE}. Comparing among fractionated O-Pec, B-Pec, and U-Pec, the spectra from protons H-1 and H-5 were different. In O-Pec (Figure 4-2) and B-Pec (Figure 4-3), the frequencies in fractions are different for H1 and H5 protons. However, in U-Pec (Figure 4-4), the frequencies of H1 and H5 protons do not change between Fractions 1-3. Fractions of O-Pec and B-Pec was variable in frequency of F_{GG} and F_{GGG} while U-Pec had more consistent F_{GG} and F_{GGG} among fractions. For the signal of EEE, all fractionated pectins had lower frequency than unfractionated pectins. This indicates that there were less contiguous blocks of ester in fractionated pectins than in unfractionated pectins. For U-Pec, the F_{EEE} frequency among fractions of U-Pec was the same except U-Pec4.

Distinguishable line patterns and the intensity of signal arise in the NMR spectra and result from different DE values and sequential arrangements of free and methyl esterified carboxylic groups along the polymer chains (Andersen and others, 1995). Denės

and others (2000) described the behavior of purified apple PME at pH7.0 and 4.5 by a combination of indirect (IEX) and direct (^{1}H NMR spectra) methods. They evaluated the frequency of F_{GGG} and F_{EEE} as a function of final DE following action of PME. The average number of successive E residues estimated the degree of multiple attack of PME. The frequencies F_{GGG}, which had higher than the Bernouillian probabilities, were considered as blockwise distribution. Andersen and others (1995) reported that a block-type distribution in enzyme treated samples is indicated by stronger lines in the spectra corresponding to contiguous arrangement of esterified and de-esterified units denoted by EE, EEEE, and GG, and corresponding weaker lines from residues characterizing block transitions, EG and GE. Grasdalen and others (1996) also reported the enzymatic reaction resulted in a high content of homogeneous triads (GGG and EEE) demonstrated the production of a sequential structure. Especially, production of a block structure enhanced the F_{GGG} fraction.

Surface Charge and Mobility by Zeta (ζ)- Potential

The ζ - Potential of O-Pec, B-Pec and U-Pec and IEX fractions in a pH range from 3 to 7 are shown in Figure 5. The negative ζ-potential increased with an increase in pH for all pectins regardless of modification. At any pH value between pH 3 and 7, O-Pec had less negative ζ-potential than U-Pec or B-Pec. The negative ζ-potential of O-Pec and B-Pec changed greatly from pH 3 to 4 and less between pH 4 –5. For U-Pec, the extent of negative ζ-potential change was greatest between pH 3 to 5 followed by slow decline. In unfractionated pectins, at any pH, the order of change in ζ-potential was O-Pec, B-Pec, and

U-Pec. The surface charge of colloids is often estimated by the ζ-potential which can be derived from the electrophoretic mobility of the particles (Anema and Klostermeyer, 1996). The measurement of the ζ-potentials yields information on the surface charge of pectin in solution at a specific pH as well as the change of the electrophoretic mobility. As pectin is acidic polysaccharides having galacturonic acid as a component sugar, the negative ζ-potential increases with pH (Nakamura and others, 2003). Nakamura and others (2003) reported the ζ-potential of soybean soluble polysaccharide (SSPS) which has a pectin like structure, pectin and their digestion products by various enzymes at pH 2-7. The negative ζ-potential of SSPS was smaller than that of pectin. Enzyme treatments increased the negative ζ-potential because the galacturonic acids which were not methylesterified were digested and lost from main backbone.

Conclusions

Valencia U-PME and P-PME de-esterify pectins which retain high MW, has more negative ζ-potential, and has different charge distributions. Based on elution of IEX, chemical shift in NMR, and ζ-potential, we observed a blockwise de-esterification pattern following a 10% decrease in DE. From elution of IEX, the peak of B-Pec and U-Pec widened and shifted to a higher ionic strength, due to increased charge and charge density, indicating blockwise action. In addition, the negative ζ-potential of B-Pec and U-Pec was greater magnitude than O-Pec at the same pH. Negative ζ-potential is enhanced by blockwise charge distribution. Based on the 2-fold increase F_{GGG} fraction, both B-PME and U-PME created blockwise de-esterification pattern. U-Pec had fewer contiguous

blocks of ester than B-Pec. Then, based on results from NMR, IEX, and ζ – potential, B-PME and U-PME has multi-attack and multi-chain pattern for modifying pectin. However, U-PME produces shorter attacks and affects more chains than B-PME.

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Table 1. Protein content and enzyme activity of Valencia PME fractions

	Protein content (μg/μl)	PME Activity (PEU/ml)	Specific Activity (PEU/mg protein)
Crude PME	0.22[a]	14.88[a]	68.29[a]
B- PME	0.05[b]	1.21[c]	25.66[c]
U-PME	0.04[b]	4.87[b]	101.31[b]

[a] Mean values with different superscripts in the same column are not significantly different at $p < 0.05$.

B-PME (SP cation exchange column -unbound and Heparin affinity column -bound PME);

U-PME (SP cation exchange column -unbound and Heparin affinity column -unbound PME)

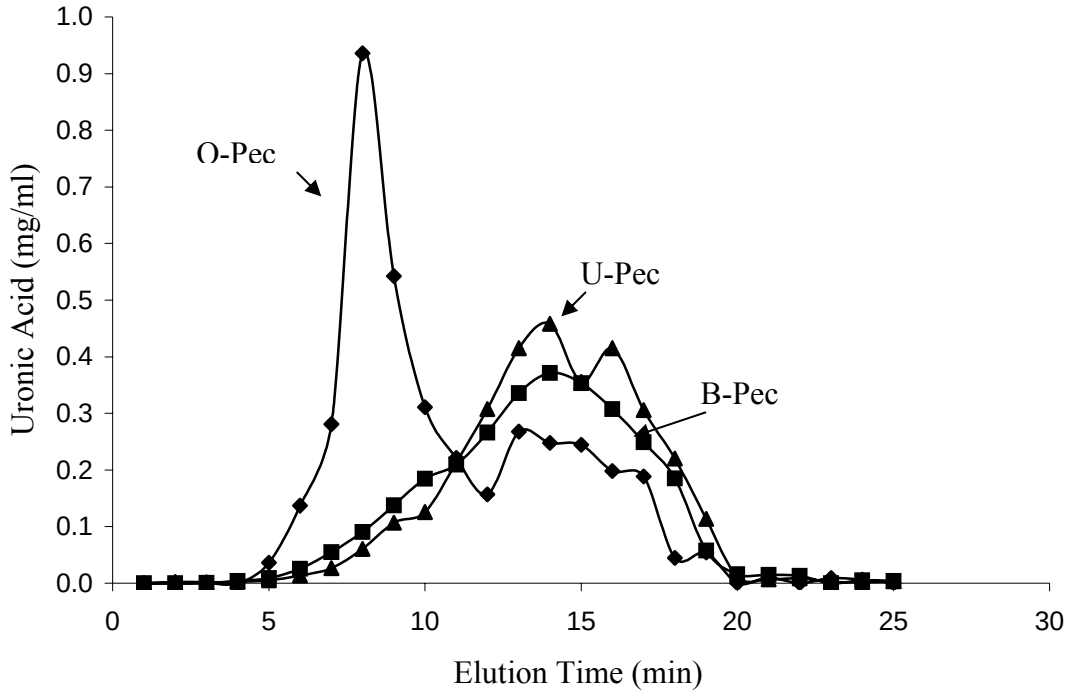

Figure 1. Analytical ion exchange chromatography elution of unfractionated pectin samples.

(O-Pec): original pectin. (B-Pec): SP-unbound and HP bound PME modified pectin. (U-Pec): SP-unbound and HP unbound PME modified pectin.

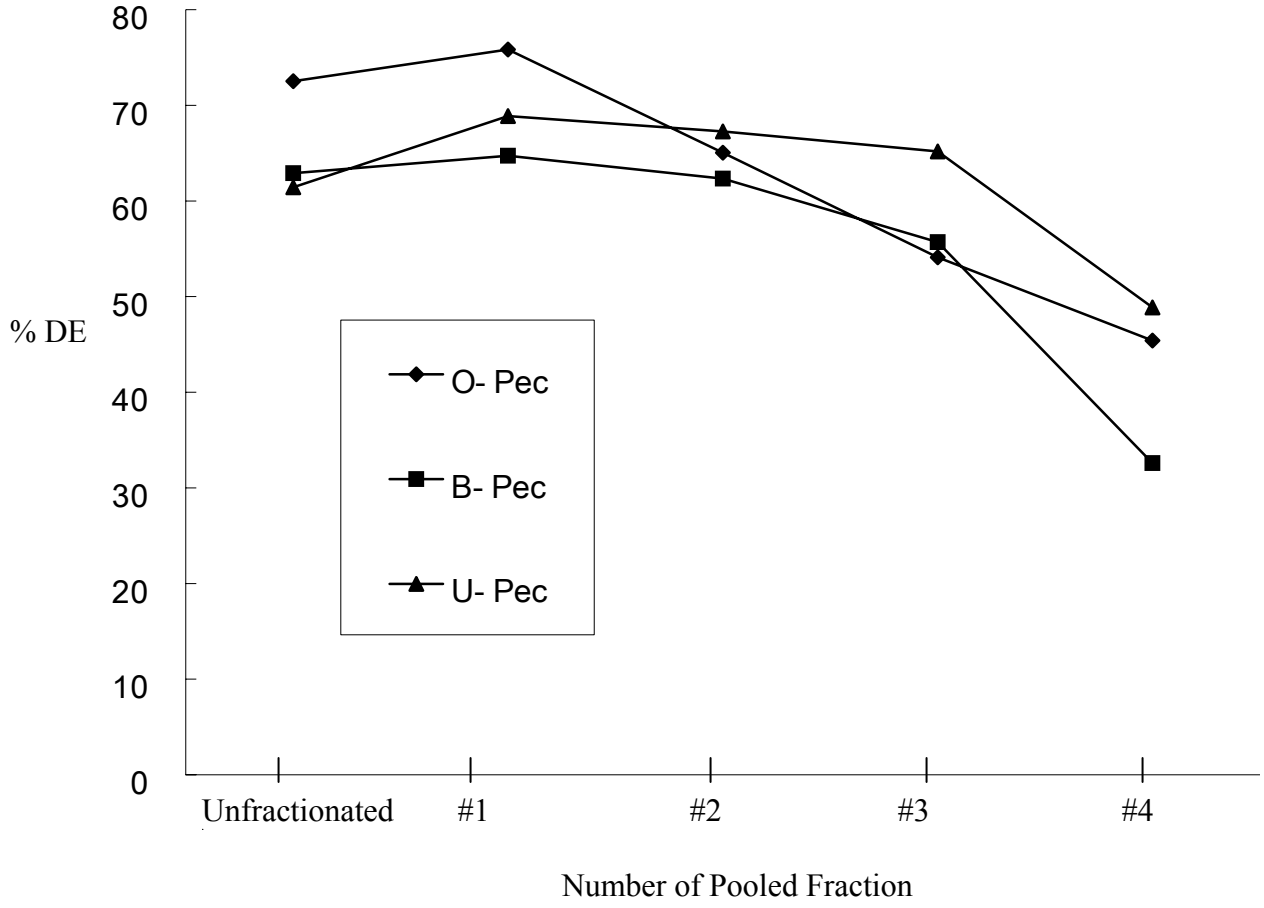

Figure 2. Degree of esterification (%DE) of unfractionated and fractionated pectin samples.

(O-Pec): original pectin. (B-Pec): SP-unbound and HP-bound PME modified pectin. (U-Pec): SP-unbound and HP unbound PME modified pectin. Fractions were pooled based on IEX elution. O-Pec2 (#90-126), O-Pec3 (#127-180), O-Pec4 (#181-216), O-Pec5 (#217-300); B-Pec1 (#50-116), B-Pec2 (#117-178), B-Pec3 (#179-251), B-Pec4 (#252-310); U-Pec1 (#60-128), U-Pec2 (#129-156), U-Pec3 (#157-203), U-Pec4 (#204-313)

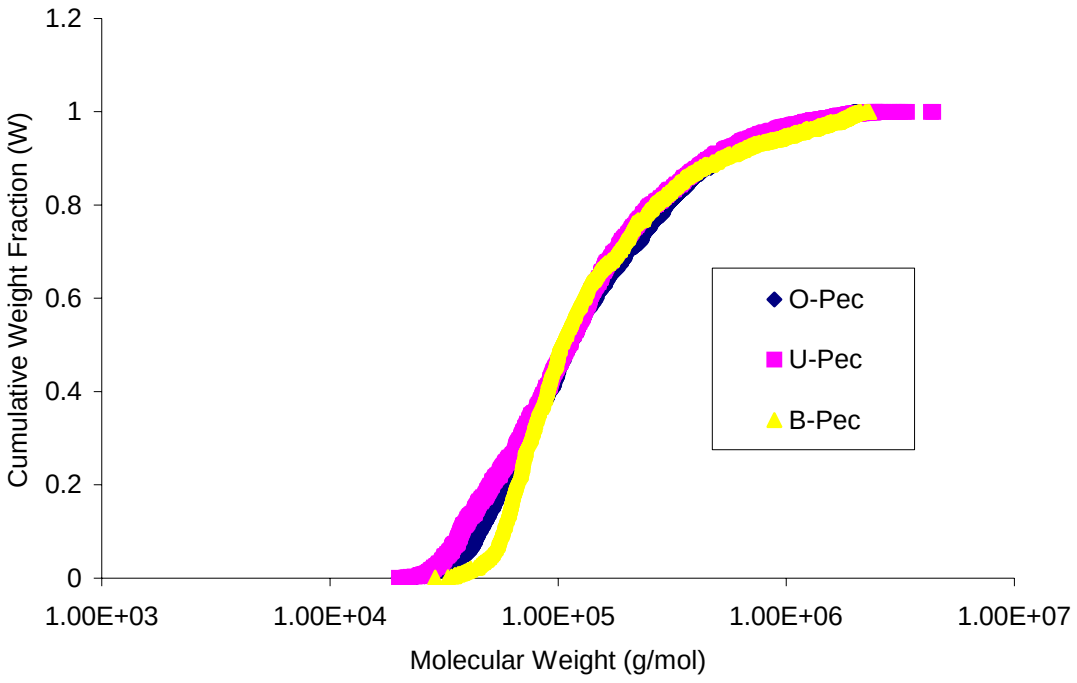

Figure 3-1. Cumulative weight fraction plotted against molecular weight of unfractionated pectin samples

(O-Pec): original pectin. (B-Pec): SP-unbound and HP bound pectin. (U-Pec): SP-unbound and HP unbound pectin.

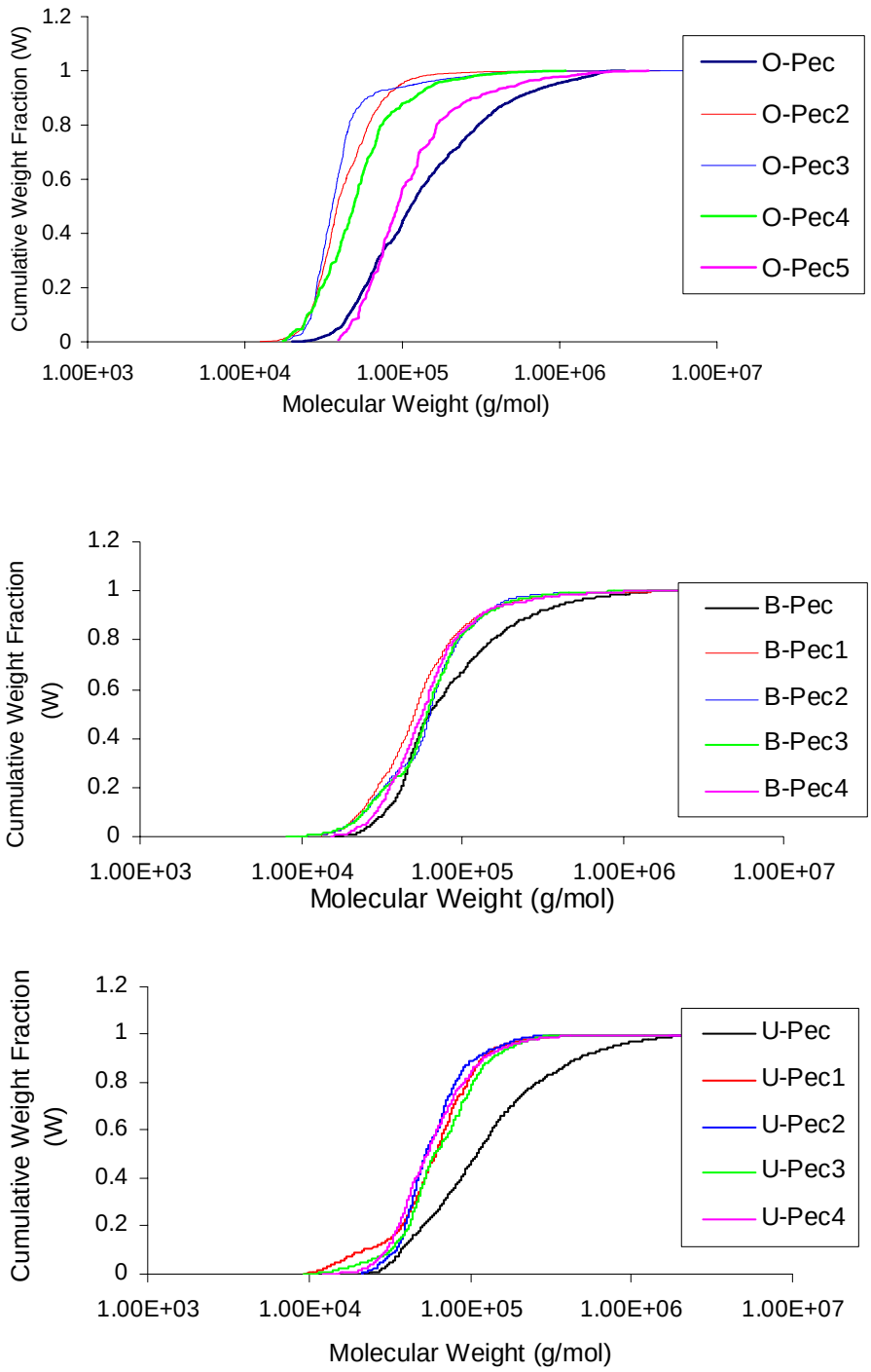

Figure 3-2. Cumulative weight fraction plotted against molecular weight of unfractionated and fractionated pectin samples.

(O-Pec): original pectin. (B-Pec): SP-unbound and HP-bound PME modified pectin. (U-Pec): SP-unbound and HP unbound PME modified pectin. Each number after sample means the pooled IEX fractionated number. For O-Pec, fraction 1 was lost.

Table 2. Monad, dyad and triad frequencies of Valencia PME modified pectins and pectic fractions at 50mM phosphate buffer, pH 7.0 in D_2O.

	Monad (H-4)		Dyad (H-1)				Triad (H-5)				
	G	E	GG	GE	EG	EE	GGG	GGE	EGG	EGE	EEE
	(4.34)	(4.39)	(5.01)	(5.06)	(4.84)	(4.89)	(4.61)	(4.58)	(4.56)	(4.54)	(4.97)
O-Pec	0.27	0.73	0.49	0.24	0.27	0.59	0.12	0.13	0.20	0.13	0.69
O-Pec2	0.24	0.76	0.44	0.26	0.31	0.65	0.05	0.11	0.15	0.05	0.66
O-Pec3	0.35	0.65	0.60	0.29	0.37	0.51	0.13	0.18	0.20	0.07	0.59
O-Pec4	0.46	0.54	0.71	0.33	0.32	0.30	0.25	0.25	0.14	0.07	0.42
O-Pec5	0.55	0.45	0.83	0.35	0.31	0.25	0.40	0.26	0.13	0.12	0.31
B-Pec	0.37	0.63	0.67	0.29	0.28	0.58	0.25	0.12	0.21	0.22	0.65
B-Pec1	0.35	0.65	0.63	0.31	0.36	0.42	0.14	0.20	0.17	0.06	0.45
B-Pec2	0.38	0.62	0.62	0.29	0.35	0.46	0.15	0.18	0.17	0.05	0.50
B-Pec3	0.44	0.56	0.71	0.26	0.32	0.39	0.27	0.16	0.14	0.03	0.45
B-Pec4	0.67	0.33	1.02	0.28	0.28	0.17	0.60	0.27	0.09	0.05	0.18
U-Pec	0.39	0.61	0.65	0.28	0.29	0.50	0.27	0.13	0.16	0.18	0.53
U-Pec1	0.31	0.69	0.57	0.30	0.33	0.48	0.10	0.19	0.18	0.04	0.52
U-Pec2	0.33	0.67	0.59	0.29	0.35	0.47	0.12	0.16	0.17	0.06	0.52
U-Pec3	0.35	0.65	0.62	0.31	0.32	0.46	0.17	0.17	0.15	0.05	0.51
U-Pec4	0.51	0.49	0.81	0.26	0.33	0.32	0.30	0.22	0.14	0.03	0.36

() means chemical shift, unit,ppm: (O-Pec): original pectin. (B-Pec): SP-unbound and HP-bound PME modified pectin. (U-Pec): SP-unbound and HP unbound PME modified pectin. Each number after sample means the pooled IEX fractionated number. For O-Pec, fraction 1 was lost. O-Pec, B-Pec, and U-Pec were repeated 2-4 times and coefficient of variation ranged from 0.23 to 3.96%. Values reported are from a single run.

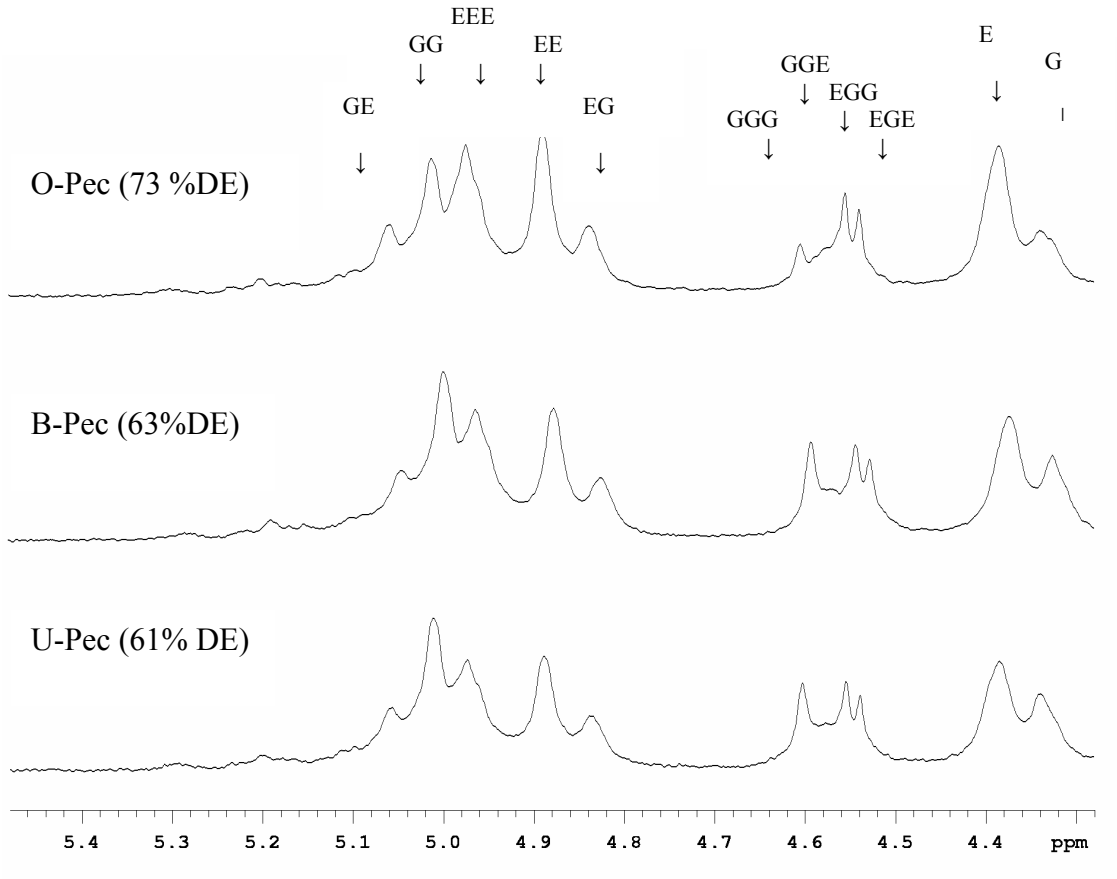

Figure 4-1. ^{1}H NMR spectra of unfractionated pectins with different degree of methyl-esterification. The O-Pec was original commercial pectin with 73%DE, and then two valencia orange PME modified pectin, B-Pec (63%DE) and U-Pec (61%DE).

(O-Pec): original pectin. (B-Pec): SP-unbound and HP-bound pectin. (U-Pec): SP-unbound and HP-unbound pectin.

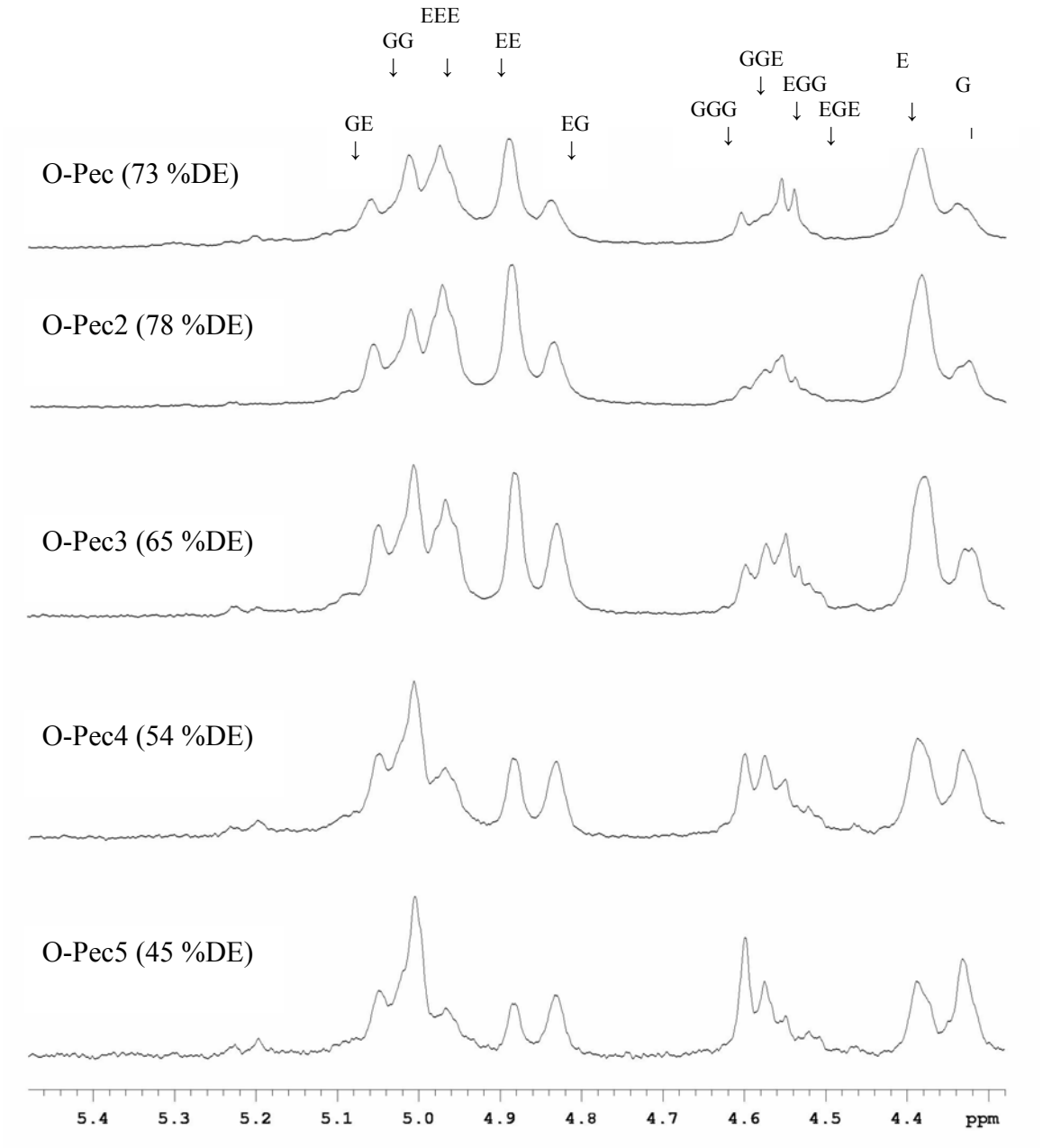

Figure 4-2. ^{1}H NMR spectra of O-Pec and its pectic fractions with different degree of methyl-esterification.

(O-Pec): original pectin. Each number after sample means the pooled IEX fractionated number. O-Pec2 (#90-126), O-Pec3 (#127-180), O-Pec4 (#181-216), O-Pec5 (#217-300)

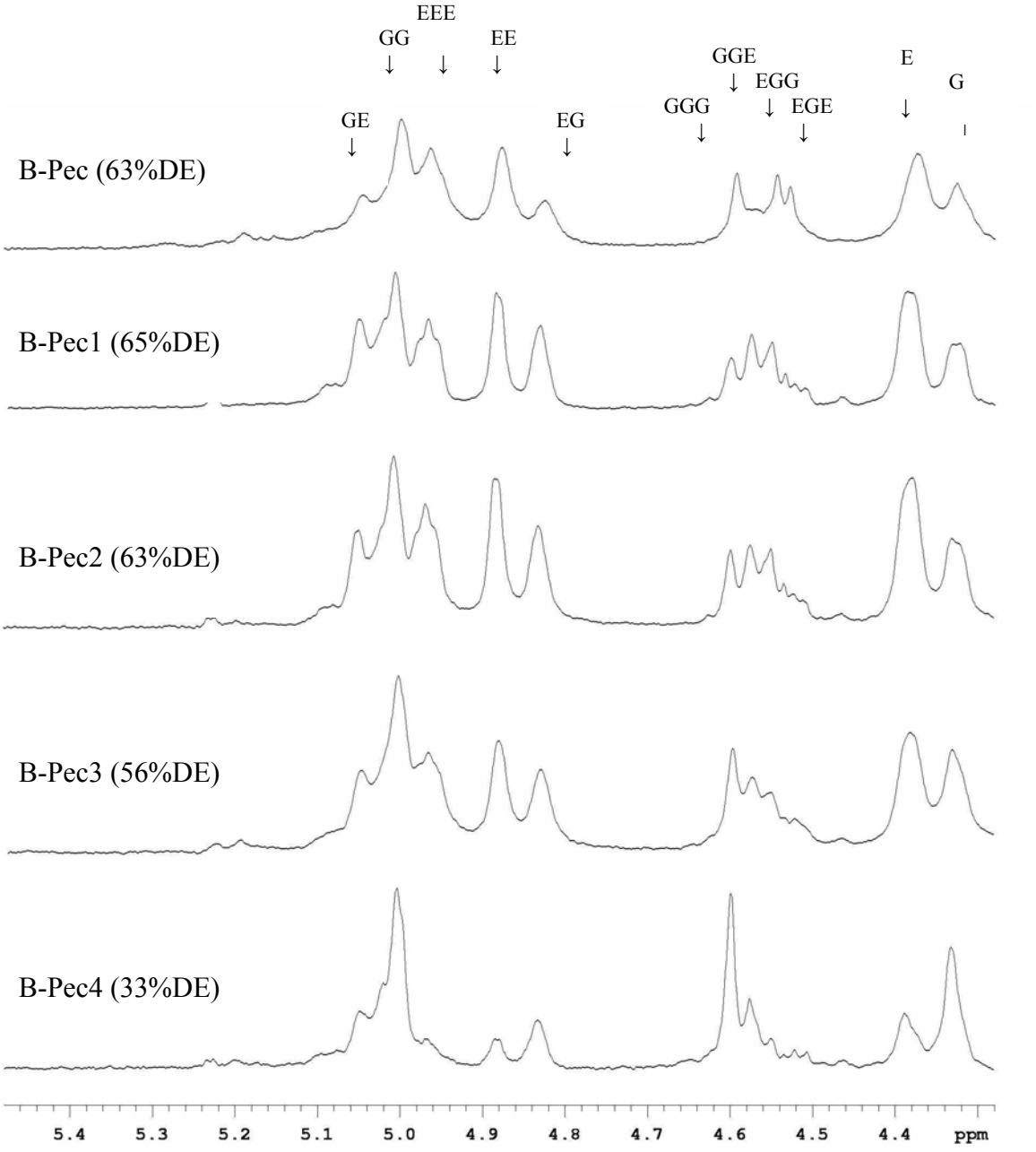

Figure 4-3. ^{1}H NMR spectra of B-Pec and its pectic fractions with different degree of methyl-esterification.

(B-Pec): SP-unbound and HP-bound pectin. Each number after sample means the pooled IEX fractionated number. B-Pec1 (#50-116), B-Pec2 (#117-178), B-Pec3 (#179-251), B-Pec4 (#252-310)

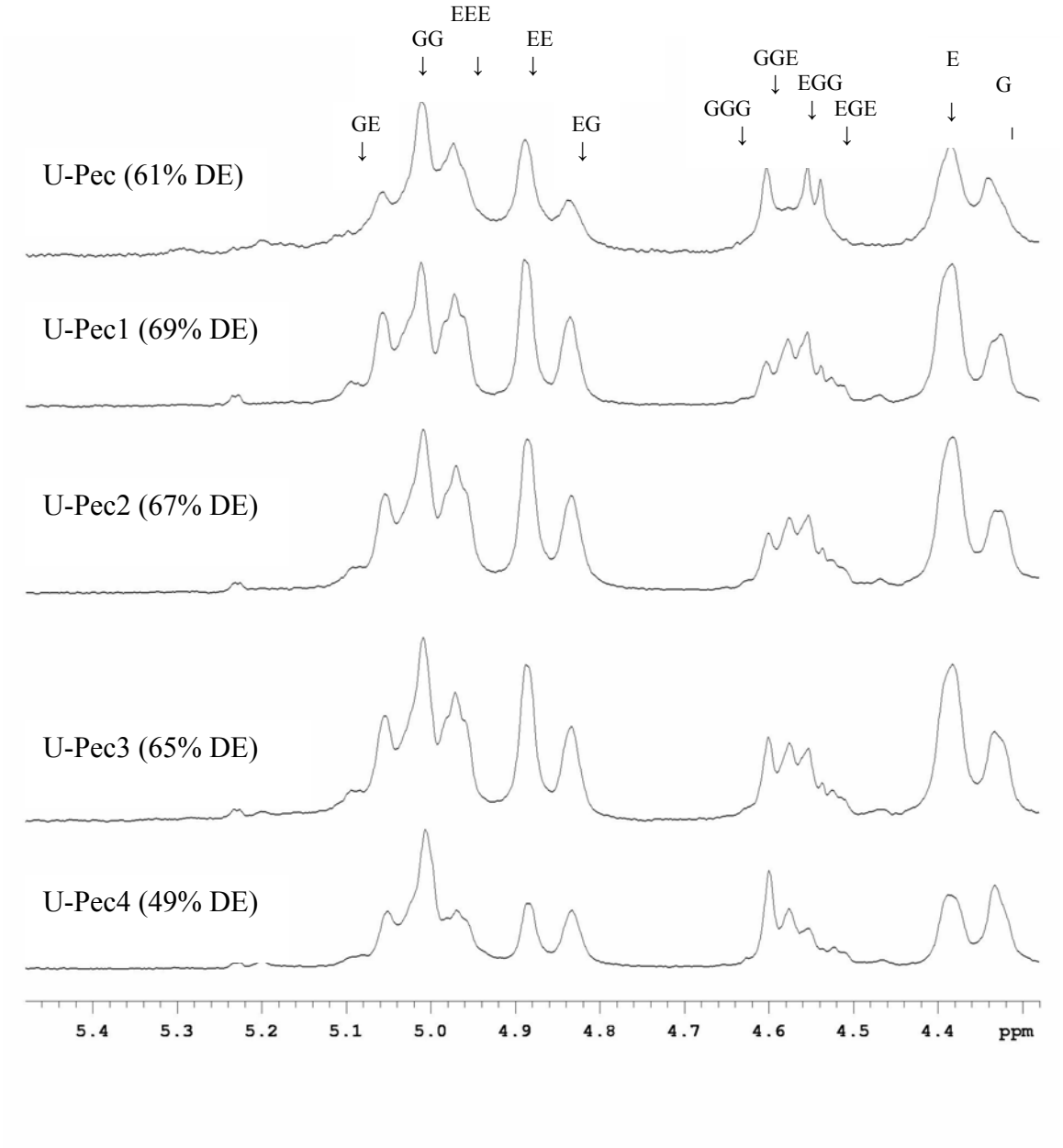

Figure 4-4. ^{1}H NMR spectra of U-Pec and its pectic fractions with different degree of methyl-esterification. (U-Pec): SP-unbound and HP-unbound pectin. Each number after sample means the pooled IEX fractionated number. U-Pec1 (#60-128), U-Pec2 (#129-156), U-Pec3 (#157-203), U-Pec4 (#204-313)

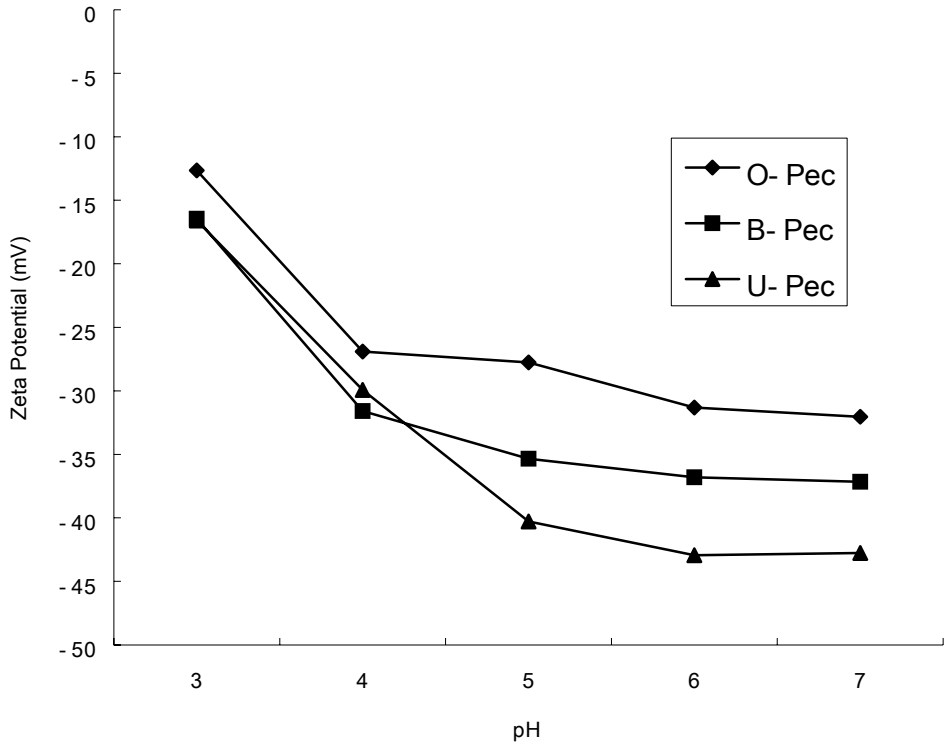

Figure 5. The zeta (ζ)-potential of 0.4% unfractionated pectin dispersion in deionized water depending on pH.

(O-Pec): original pectin. (B-Pec): SP-unbound and HP-bound pectin. (U-Pec): SP-unbound and HP-unbound pectin.

CHAPTER 4

CALCIUM SENSITIVITY OF VALENCIA PME MODIFIED PECTINS BY ζ- POTENTIAL, TEXTURAL AND RHEOLOGICAL PROPERTIES[2]

[2] Kim YK, Wicker L. To be Submitted to *Food Hydrocolloids*, 2004

Abstract

Calcium sensitivity of unmodified pectin (O-Pec) and two Valencia PME modified pectins (B-Pec and U-Pec) were investigated before and after fractionation by IEX based on the ζ-potential, TPA and rheological measurements. U-Pec and B-Pec had more negative ζ-potential than O-Pec, and became more negative at higher pH values. $CaCl_2$ led to pectin dispersions with less negative ζ-potential. The viscosity of O-Pec in absence of $CaCl_2$ was higher than B-Pec and U-Pec, and all samples displayed slightly non-Newtonian behavior. In the presence of 35 mM $CaCl_2$, 2% B-Pec and U-Pec formed a gel, in contrast to O-Pec. No significantly different texture profile was observed between B-Pec and U-Pec. At 2%, U-Pec or B-Pec exhibited solid like behavior with $CaCl_2$. O-Pec had 20 or 50 fold lower G' than B-Pec and U-Pec. At 0.4 Hz, the G' values of U-Pec, B-Pec and O-Pec showed 503 Pa, 219 Pa and 9 Pa, respectively. Fractionated pectins had lower viscosity and G' than unfractionated pectins regardless of modification. TPA and G' results for U-Pec and B-Pec are consistent with unique pattern of de-esterification that provide a unique charge distribution and population of pectin that is de-esterified.

Key words: multiple attack, multiple chain, mechanism of de-esterification, gelling, viscosity

Introduction

Pectins are important structural polysaccharides in the cell walls of many plants which are of considerable interest as gelling agent in food industry (Nelson and other, 1977; Voragen and others 1995). Basically, they consist of a linear backbone of α (1-4)-D-galacturonic acid residues partially esterified with methanol, with periodic interruptions to L-rhamnose residues 1,2-linked that make the backbone irregular, and with some other neutral sugars present as side chains (De Silva and others 1995).

Their methyl ester group content, expressed as degree of methyl esterification (DE), implies a specific gelling mechanism. High-methoxyl pectin (HMP, >50% DE) requires low pH (~3.5) and the addition of water soluble solute, typically sucrose, for gelation through hydrogen bonds and hydrophobic interaction. The affinity HMP for Ca^{2+} is generally not high enough for sufficient chain association and gelation. Low-methoxyl pectins (LMP, <50% DE) gel through the ionic interactions of polyvalent cations, such as Ca^{2+}, in the absence of sucrose (Gilsenan and others 2000). Normally, the interaction of Ca^{2+} increases with decreasing DE and a transition in calcium affinity occurs around a 40 %DE (Ralet and others 2001).

Besides the methoxy content, the distribution pattern of free and esterified carboxyl groups and the length of unsubstituted galacturonan backbone have an effect on the strength of calcium binding. LMPs with similar ester content prepared by different de-esterification procedures have different gelling properties because of the different distribution of free carboxyl groups along the polygalacturonic acid chain (Heri and others,

1961; Kohn and others, 1968). Chemical de-esterification is a random process that can result in decreased molecular weight due to de-polymerization of pectin backbone by β-elimination. However, enzymatic de-esterification results in a blockwise distribution and undesired depolymerization of pectins is reduced (Gemeiner and others, 1991; Limberg and others, 2000). Especially, plant pectin methylesterase (PME, E.C. 3.1.1.11) can create a calcium sensitive pectin (CSP) in which HMP can gel in the presence of Ca without the addition of sucrose as long as blocks of de-esterified pectin are present (Joye and Luzio, 2000). Liner and Thibault (1992) suggested that a minimal block size of nine de-esterified residues was necessary for calcium cross-linking and hypothesized that a larger de-esterified block might be necessary for gel formation. Larger blocks were found in the enzyme de-esterified pectin than in the alkali and acid de-esterified material (Tuerena and others, 1982). Willats and others (2001) found that the degree and pattern of methyl-esterification affects the elasticity of the gels as well as their response to compressive strain. For enzymic (blockwise) de-esterification, the extent of Ca^{2+} binding increased almost linear relationship with free carboxyl groups, whereas chemical (random) de-esterification showed a non-linear relationship of a form consistent with the requirement of this binding for blocks of contiguous non-esterified residues (Powell and other, 1982). Ralet and others (2003) also showed that the blocks formed by plant-PME treatment allowed blocks to form calcium-pectinate precipitates for high DE even though these blocks were not long enough to induce abnormal polyelectrolyte behaviour. Cameron and others (2003) reported a 6.5 % de-esterification by a Valencia PME increased the calcium sensitivity without a decrease in

Mw. Kohn and Luknar (1975), reported that pectins de-esterified by plant PMEs exhibited calcium binding properties close to that of polygalacturonic acid up to a DE of at least 60% because the number of contiguous unesterified galacturonic acid residues is needed to form stable junction zones. Moreover, the gelling capacity in the presence of calcium is also dependent on other intrinsic and extrinsic parameters, like the charge distribution along the backbone, the number and size of side chains, the average molecular weight, the ionic strength, the pH, the temperature, and the presence of cosolutes (Garnier and others, 1993; Voragen and others, 1995).

The impact of structural change from different PME modification on functional properties of pectins has been investigated by several physicochemical methods. These include equilibrium dialysis and calcium activity determinations, mainly as a function of the degree of polymerization of the pectin and the methoxy content (Thibault and Rinaudo, 1986; Hotchkiss and others, 2002; Schmelter and others 2002; Joye and Luzio, 2000). These studies have been carried out on dilute solutions by the determination of calcium activity coefficients and on calcium gels mainly by measurements of the gel strengths (Powell and others, 1982). In earlier research (Kim and others, 2004), unique Valencia PMEs were used to generate modified pectins (U-Pec and B-Pec) that had the same total charge but differed in charge distribution. The objective of this research was to characterize the calcium sensitivity of Valencia PME modified pectin by direct measure of viscous and gelling properties in the presence of $CaCl_2$. In addition, the effect of calcium on the surface charge of the modified pectins was estimated by measuring the ζ-potential.

This research has application to the development of tailored pectins for specific functional properties using PMEs of known mechanism of action.

Materials and Methods

Materials

As described in a previous study (Kim and others, 2004), commercial, unstandardized high methoxyl pectin (citrus pectin type 104, CP Kelco, Svenved, Denmark) was modified by Valencia PME extracts (U-PME and B-PME) and fractionated into 4 fractions using preparative ion exchange chromatography (IEX, Q5 anion exchange). The fractions were dialyzed against deionized water, freeze dried (Unitop 600L, Freeze Mobile 25SL, VisTris, Gardiner, NY), ground and stored at -20°C until further study.

Zeta (ζ)- Potential

A dispersion of 0.4% (w/w) pectin with or without 10 mM $CaCl_2$ was adjusted to pH values between 3 and 7 by HCl and NaOH. Measurement of the ζ- potential was performed by a Particle Size Analyzer adding the BI-Zeta option (90 Plus, Brookhaven Inst., Holtsville, NY) with a 50 mW diode laser (90 angle) and a BI-9000AT correlator. All experiments were carried out at 25°C with the laser beam at 659.0 nm and 1.330 as the refractive index. The ζ- potential was determined in triplicate with 10 runs subsequently after the particle size determination for the same sample of pectin solution.

Texture Profile Analysis (TPA) of Pectin Gels

Pectin gel was prepared a modified method of MacDougall and others (1996). A 500 mM $CaCl_2$ solution was added to 2% pectin disperion to a final concentration of 35

mM at 60°C. The mixture was immediately mixed by a vortex, stored 24 h at 4°C. After the gels were tempered to room temperature, the texture profile was measured (TA-XT2i, Texture Technologies Corp, Scarsdale, NY, fitted with a 25 kg load cell). The settings and operation of the instrument were accomplished using Texture Expert software version 2.12™ (Texture Technologies, Scarsdale, New York, U.S.A.). Textural properties including hardness, cohesiveness, adhesiveness, gumminess, and chewiness were calculated from the curve according to definitions given by Bourne (1978). Samples were compressed to 30% of the initial height. The pre-test, test, and post-test speeds were set to 2, 2, and 2 mm s^{-1}, respectively.

Rheological Measurement

The viscosity of pectin was determined using a Controlled Stress Dynamic Rheometer™ (Rheometrics, Piscataway, New Jersey, U.S.A.) equipped with a cone and plate device (60 mm diameter, 0.0385°, 0.4 mm gap). Flow curves of 2 % pectin dispersion were increased by shear rate (10 - 50 s^{-1}) at 20 °C. Shear rate against shear stress data were fit using the power law model, and analyzed for the flow behavior, n and consistency index, k. For the viscoelastic properties of pectin gels, gelation was induced directly on the rheometer plate by mixing 1ml of 2% pectin and stock 500 mM $CaCl_2$ solution to a final concentration of 35 mM and pre-shearing for 30s at 5 Pa. After a 10 min equilibration, storage modulus G' and loss modulus G" was measured as a function of time at a frequency of 1 Hz and a stress of 1 Pa. The stress applied was 1 Pa, which was verified to be in the linear regime. The gels were further characterized by measuring between 0.1 and 6 Hz at 1

Pa. The Dynamic Stress Sweep Test, at fixed frequency of 1 Hz, was also performed for the viscoelastic properties with an initial and final stress of 0.1 Pa to 10 Pa respectively. All rheological experiments were conducted at 20ºC.

Statistical Analysis

All experiment was done by three replicates. The difference of means among the samples for TPA and viscosity factors were analyzed by the general linear model procedure (SAS program version 6.12™, SAS Institute Inc., Cary, North Carolina, U.S.A.) using a Duncan test at the level of significance ($p=0.05$).

Results and Discussion

Sample Characterization Summary

The chemical characteristics of pectins used in this study are described by Kim and others (2004) and summarized in Table 1. Original pectin (O-Pec, 73 %DE) was de-esterified by Valencia PMEs to create two modified HMP (U-Pec, 61 % DE and B-Pec, 63 % DE). B-Pec and U-Pec have similar total DE values, Mw and polydispersity after modification. However, Valencia PME modification created more negative ζ-potential. U-Pec had more negative ζ-potential than B-Pec. Based on IEX elution, ζ-potential, and NMR analysis of IEX fractions, Kim and others (2004) concluded that Valencia PME resulted in multichain, blockwise attack. The pattern of de-esterification in U-Pec was shorter blocks and affected a greater proportion of the pectin populations. In B-Pec, the blockwise de-esterification was longer, but affected fewer pectin molecules.

Zeta (ζ) - Potential

The ζ - Potential of O-Pec, B-Pec and U-Pec and IEX fractions with and without 10 mM $CaCl_2$ in a pH range from 3 to 7 are shown in Table 2. As an acidic polysaccharide, the negative ζ- potential of pectin increases in pH (Nakamura and others (2003). In absence of $CaCl_2$, the negative ζ-potential increased with pH for all pectin samples and was greater in U-Pec and B-Pec than O-Pec. The addition of $CaCl_2$ to pectin dispersions created less negative ζ-potential at all pH values. In this case, B-Pec and U-Pec had similar negative ζ-potential at all pH values, but still lower than O-Pec.

In fractionated O-Pec, B-Pec, and U-Pec, a loss of negative charge with calcium chloride addition was observed, especially at lower pH values less than pH 5. For O-Pec, there was similar a negative ζ-potential regardless of fraction number at pH values greater than 5. However, in the presence of $CaCl_2$ at pH values greater than 5, the charge of early eluting B-Pec fraction 1 was markedly less than later eluting fractions of B-Pec. This is likely the result of masking of slight charge in the early eluting fraction of B-Pec. In the presence of $CaCl_2$ at pH values greater than 5, U-Pec fraction 2 had lower charge than earlier or later eluting fractions.

Nakamura and others (2003) reported the ζ-potential of soybean soluble polysaccharide (SSPS) which has a pectin like structure, pectin and their digestion products by various enzymes at pH 2-7. The negative ζ-potential of SSPS was smaller than that of pectin. Enzyme treatments increased the negative ζ-potential because the galacturonic

acids which were not methylesterified were digested and lost from main backbone. Kulmyrzaev and others (2000) mentioned some minerals bind to oppositely charged groups on the surface of whey protein emulsion droplets, decreasing the magnitude of their ζ-potential and thereby reducing the electrostatic repulsion between droplets. The ζ-potential of gum arabic stabilized oil in water has been investigated in different concentration of NaCl over a pH range from 1 to 10 (Jayme and other ,1999). The trends observed showed decrease in negative ζ-potential with increasing salt concentration since an increase in salt concentration will lead to a compression of the electrical double layer and a corresponding reduction on ζ-potential. Here, pectin dispersion also showed a similar tendency as whey emulsion system with addition of minerals. In two modified pectins, block-structures on the homogalacturonan backbone made from PME de-esterification allow $CaCl_2$ crossing linking of pectin chains, thus surface charge could be reduced compared with unmodified pectin dispersion.

Texture Profile Analysis of 2% Pectin Gel

The O-Pec did not form a gel in the presence of 35 mM $CaCl_2$, while B-Pec and U-Pec formed clear elastic gels and 30% recovered from small static deformation on removal of the applied stress. There was no significant difference in neither hardness nor other texture profiles between U-Pec and B-Pec gels, 0.88N ± 0.30 or 0.80 N± 0.07, respectively ($p>0.05$) (Table 3).

The gelling properties of modified pectins after IEX fractionation varied. For B-Pec and U-Pec, early eluting fractions 1 or 2 thickened, but gel strength could not be measured.

Later eluting U-Pec fractions 3 and 4 and B-Pec fraction 3 formed a measurable gel. For B-Pec fraction 4, it didn't form a measurable gel even if it was a later eluting fraction. All fractions of U-Pec or B-Pec had significantly lower texture profile values than unfractionated U-Pec or B-Pec gels. Among the fractionated pectin gels, there was no difference. The lower hardness and TPA parameters of the modified pectin fractions may be related to the lower MW reported for fractionated pectins. Presumably, early eluting pectic fractions may contain a minimal block size for calcium cross-linking, but not sufficient for gel formation (Liner and Thibault, 1992).

The degree and pattern of methyl-esterification affects the elasticity of the gels and response to compressive strain. Lower DE pectins typically form stronger gels than high DE pectins. In the case of pectins with similar DE values, but different distribution pattern, a nearly 3-fold increase in yield point of pectin gels modified by plant PME was observed compared to pectins modified by alkali (Willats and others, 2001). For calcium sensitive (CS) and non-calcium sensitive (NCS) pectins, the CS pectin gels are more weak and distortable than NCS gels (Laurent and Boulenguer, 2003).

Viscosity of 2% Pectins in the absence of $CaCl_2$

Since TPA was not possible on O-Pec and some fractions of modified pectins, the viscosity of pectins was evaluated to provide insight into structural changes related to PME de-esterification. In the absence of $CaCl_2$, unfractionated pectins displayed shear-thinning behavior regardless of modification. The n values of 2% O-Pec and U-Pec were not different at 0.91 and 0.91 while the n values of 2% B-Pec was different at 0.96, ($p<0.05$).

Marcotte and others (2001) also reported that pectin dispersions exhibited a power-law shearing thinning behavior, characterized by an n value less than 1 at concentrations range 1% to 5% and temperature range 20°C to 60°C. However, the k values of O-Pec, B-Pec, and U-Pec were significantly different ($p<0.05$) and decreased in the order of O-Pec, U-Pec and B-Pec, 0.14, 0.09, 0.03, respectively.

For unfractionated pectins, the viscosity of O-Pec (0.08 $Pa \cdot S^{-1}$) was higher than B-Pec (0.03 $Pa \cdot S^{-1}$) or U-Pec (0.06 $Pa \cdot S^{-1}$) over the entire shear rate range. Viscosity is affected by several factors, including molecular weight, aggregation, conformation of molecules, degree of esterification, pattern of esterification, pH, temperature, and pectin sources (Li and Chang 1997; Morris and others 2002: Marcotte and others 2001). The viscosity of unfractionated or fractionated O-Pec, B-Pec, and U-Pec at a shear rate range between 10 and 280 Pa is depicted in Figure 1. All viscosity of fractionated pectin was lower than unfractionated pectins regardless of modification. It seems to be related to the tendency in Mw. That is, fractionated pectins showed significaly decrease in Mw comparing to unfractionated pectins after IEX fractionation due to some loss, because highly de-esterified pectin aggregation may be stuck in column during the fractionated IEX (Kim and others, 2004). Fractions of B-Pec or U-Pec showed higher viscosity than fractions of O-Pec, even if unfractionated O-Pec was more viscous than B-Pec or U-Pec. It may indicate that PME modification lead fractionated pectins to get more viscosity. It is also in the agreement to the results in Mw and effective diameter of pectins. For example,

O-Pec fractions had smaller Mw values than B-Pec and U-Pec fraction. The range of viscosity in U-Pec showed more narrow than range of B-Pec or O-Pec.

Schmelter and others (2002) reported that enzymatic alternation of the side chain regions yielded a significantly lower viscosity in the absence calcium, while the viscosity of calcium-free pectin samples was increased after de-esterification of the backbone with PME. Hotchkiss and others (2002) reported PME treated pectin occurring %DE 70 to 32 had a 16% reduction in intrinsic viscosity (IV) with no reduction in Mw using high-performance size exclusion chromatography with on-line multiangle laser light scattering. In contrast, alkali deesterification rapidly reduced both Mw and IV to less than half of that observed for untreated pectin. Thibault and Rinaudo (1985) showed no decrease in IV values of pectin with DE about 10, 30, and 40% after enzyme deesterification by determinating the calcium activity coefficients. Compared to unfractionated pectins, pectic fractions had smaller Mw. This may be due to high viscosity of the concentrated pectin samples resulted in elution of part of the pectin at IEX by the ions present in the injected sample (Schols and others, 1980). Thus, the low capacity of cartridge resulted in the procedure not reproducible and low recovery after IEX separation. There also might be some loss because highly de-esterified pectin aggregation may be stuck in column during the fractionated IEX.

Viscoelastic Properties of Pectins with $CaCl_2$ based on Three Kinds of Factors

Over all frequency ranges, 1% pectin dispersion with 35 mM $CaCl_2$ exhibited a more solid like behavior with G' > G" and slowly increased according to frequency (Table

4). Pectins gelled in presence of $CaCl_2$ except all O-Pec fractions at 0.4 Hz. G' values for O-Pec were 20 or 50 fold lower than that for B-Pec or U-Pec after addition of $CaCl_2$. At 0.4 Hz, the G' values of U-Pec, B-Pec and O-Pec showed 502 Pa, 219 Pa and 9 Pa, respectively. Fractionated pectins had lower G' values than the unfractionated pectins. Later eluting IEX fractions usually had higher G' value than earlier eluting fractions. Usually, U-Pec and its fractions had higher G' values than other samples. B-Pec3 had the highest G' value of the fractionated pectins and G' decreased sharply in B-Pec2 or B-Pec4. On the other hand, G' values of fractions of U-Pec increased from Fraction 2 to Fraction 3 and remained the same at Fraction 4. These results support that U-Pec and B-Pec have a different pattern of de-esterification.

The G' values of pectin dispersions after addition of 35mM $CaCl_2$ at 1 Pa stress and 1 Hz frequency, are depicted in Figure 2 for the three unfractionated pectins and selected modified, fractionated pectins. Over time, the G' values did not change. At 1500 sec, the G' values of U-Pec, B-Pec and O-Pec were 544 Pa, 326 Pa and 11 Pa, respectively. Fractions of pectin had lower G' values than unfractionated pectins in all cases. Pectin fractions of U-Pec and B-Pec were higher than fractions of O-Pec.

Norziah and others (2001) investigated the viscoelastic properties of HMP dispersions at pH 3.0 depending on varying concentrations, sucrose and calcium. Increasing pectin, sucrose and calcium concentrations increased G' and G" with pectin having the greatest effect. Dispersions of pectin alone or in combination with sucrose exhibited a more liquid-like behaviour with G" > G'. However, in the presence of Ca^{2+}, mechanical

spectra of G'>G" were obtained. Lopes da Silva and others (1993) reported that LMP and HMP dispersion showed a quite different behavior of dynamic rheological properties caused by the higher charge density in LMP which was related to a lower intermolecular association and a higher hydrodynamic volume. Schmelter and others (2002) reported that the G' values of de-methoxylated pectin was increased 35-fold and the gel-like properties were markedly enhanced in the presence of calcium. At the rheological data for the unmodified (64 %DE) and the reduced pectin (43% DE) (Rosenbohm and others, 2003), the unmodified pectin showed no elasticity (G') at low concentration or lack of a values for G' even at the high concentration. However, the reduced pectin showed the G' values was greater than G" indicating gel-like behavior even for the lowest concentration. Ralet and others (2003) suggested that extensive pectin de-methylation causes pectin precipitation with calcium, as is the case when multiple regions of predominant free galacturonic acid groups are present on the same pectin. That is, high ester pectin with regions of free galacturonic acid groups can form multiple calcium bridges, which create a domain of strong, intermolecular association between the galacturonan chains. In the presence of calcium, increased viscosity and gelling may result (Schmelter and others, 2002).

Conclusion

This study of the calcium sensitivity of Valencia PME modified pectins led to a better insight into the effect of different pattern of ester distribution on functional properties of B-Pec and U-Pec. In the presence of $CaCl_2$, the ζ-potential, TPA, and rheology studies confirm the difference between B-Pec and U-Pec as well as fractioned pectins. B-Pec and

U-Pec in the presence of $CaCl_2$, showed 20 or 50-fold higher G' than O-Pec, while O-Pec had higher viscosity than B-Pec or U-Pec in the absence of $CaCl_2$. A similar result was observed in fractionated pectins. Moreover, fractionated pectins showed lower viscosity and lower G' than unfractionated pectins regardless of modification, most likely related to lower MW. Based on results from ζ-potential, TPA, and rheology study, we conclude that B-Pec and U-Pec, with similar ester content, have different gelling properties because of the different distribution of free carboxyl groups along the polygalacturonic acid chain. Most likely, U-Pec results from multiple attack by PME for a larger pectin population and B-Pec also results from multiple attack over longer blocks of fewer pectin molecules.

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Table 1. The summary of chemical properties of O-Pec, B-Pec, and U-Pec by Valencia PME[a].

	% DE [b]	Mw (g/mol)	Polydispersity (Mw/Mn)	ζ-potential (mV)
O-Pec	73	134,000 ± 3,439	1.96	-21.36 ± 0.14
B-Pec	63	132,250 ± 3,889	2.13	-30.10 ± 1.62
U-Pec	61	133,850 ± 3,748	2.11	-39.67 ± 1.05

[a] Data adapted from Kim and others, 2004
[b] % DE from NMR spectra. Coefficient of variation ranged from 0.23 to 3.96% for 2 to 4 replicates. Mw: weight average molecular, Mn: number average molecular weight (O-Pec): original pectin. (U-Pec): SP-unbound and HP unbound PME modified pectin. (B-Pec): SP-unbound and HP bound PME modified pectin.

Table 2. ζ potential of 0.4% pectin solution with and without 10 mM $CaCl_2$ over a pH range from 3 to 7. (unit: mV)

	Without $CaCl_2$					With $CaCl_2$ (Final conc. 10mM)				
Sample	pH 3	pH4	pH5	pH6	pH7	pH 3	pH4	pH5	pH6	pH7
O-Pec	-12.64 ± 1.05	-26.91 ± 1.05	-27.77 ± 1.98	-31.31 ± 1.15	-32.04 ± 1.78	-5.41 ± 1.03	-13.9 ± 1.37	-12.62 ± 1.37	-13.32 ± 1.12	-14.87 ± 0.66
O-Pec2	-20.62 ± 3.38	-23.1 ± 0.97	-29.67 ± 1.26	-31.59 ± 1.14	-31.43 ± 0.76	-6.42 ± 0.73	-10.02 ± 1.65	-13.16 ± 0.34	-17.64 ± 0.22	-11.63 ± 1.65
O-Pec3	-9.39 ± 2.04	-17.31 ± 1.03	-16.49 ± 0.00	-27.35 ± 0.15	-23.06 ± 3.73	-8.35 ± 2.81	-12.96 ± 3.22	-2.96 ± 1.88	-13.31 ± 1.79	-15.8 ± 0.79
O-Pec4	-12.3 ± 3.34	-21.39 ± 5.54	-25.16 ± 4.97	-29.44 ± 1.38	-30.2 ± 1.80	-1.68 ± 1.45	-9.17 ± 4.16	-14.51 ± 1.74	-15.24 ± 1.54	-11.95 ± 5.70
O-Pec5	-8.99 ± 0.99	-23.42 ± 0.60	-22.27 ± 6.33	-27.23 ± 5.51	-22.71 ± 4.94	-6.88 ± 0.47	-14.54 ± 0.51	-14.24 ± 3.13	-19.78 ± 2.66	-14.48 ± 1.13
B-Pec	-16.45 ± 1.35	-31.58 ± 0.08	-35.35 ± 1.75	-46.51 ± 0.76	-37.16 ± 0.65	-9.77 ± 0.77	-10.23 ± 2.75	-12.79 ± 1.12	-21.09 ± 1.68	-12.99 ± 0.90
B-Pec1	-3.87 ± 1.70	-15.21 ± 4.03	-19.4 ± 1.64	-14.23 ± 2.72	-15.1 ± 2.81	0.78 ± 0.99	-7.32 ± 5.47	-1.28 ± 1.06	-6.85 ± 4.57	-9.42 ± 3.90
B-Pec2	-7.37± 1.28	-23.35 ± 1.44	-28.47 ± 2.08	-17.33 ± 0.96	-25.29 ± 3.35	-1.41 ± 1.19	-7.94 ± 4.02	-14.73 ± 1.77	-11.33 ± 4.00	-12.14 ± 0.24
B-Pec3	-8.23 ± 4.67	-18.41 ± 3.83	-20.1 ± 9.75	-15.53 ± 11.46	-15.48 ± 1.07	-10.11 ± 3.08	-17.73 ± 1.26	-13.87 ± 2.74	-10.19 ± 0.01	-13.09 ± 0.00
B-Pec4	-8.32 ± 1.11	-28.53 ± 1.59	-30.34 ± 4.33	-20.25 ± 4.43	-16.41 ± 2.28	-7.71 ± 4.30	-15.25 ± 0.73	-19.63 ± 0.58	-12.32 ± 1.13	-14.24 ± 4.40
U-Pec	-16.59 ± 6.84	-29.93 ± 1.91	-40.29 ± 0.64	-42.94 ± 1.01	-42.76 ± 0.71	-6.76 ± 0.67	-11.85 ± 1.18	-15.61 ± 1.02	-12.64 ± 1.28	-13.68 ± 0.03
U-Pec1	-7.8 ± 1.56	-20.9 ± 6.83	-26.55 ± 4.11	-26.64 ± 6.51	-17.11 ± 0.06	-5.06 ± 3.33	-7.46 ± 1.42	-13.1 ± 4.56	-12.03 ± 2.17	-14.58 ± 1.80
U-Pec2	-7.84 ± 0.20	-7.71 ± 2.51	-9.83 ± 3.15	-28.22 ± 6.30	-19.56 ± 4.16	-5.37 ± 0.84	-3.59 ± 3.26	-6.41 ± 1.88	-7.65 ± 6.48	-13.03 ± 3.61
U-Pec3	-9.83 ± 2.89	-14.96 ± 6.80	-24.15 ± 1.63	-18.07 ± 0.04	-34.33 ± 0.24	-5.21 ± 4.99	-11.99 ± 4.34	-14.76 ± 1.70	-14.92 ± 1.43	-15.8 ± 0.59
U-Pec4	-12.13 ± 1.26	-26.63 ± 1.53	-29.8 ± 2.81	-36.89 ± 1.53	-31.14 ± 1.34	-3.19 ±1.09	-14.92 ± 6.80	-12.63 ± 2.13	-17.18 ± 0.22	-16.78 ± 1.03

(O-Pec): original pectin. (B-Pec): SP-unbound and HP bound PME modified pectin. (U-Pec): SP-unbound and HP unbound PME modified pectin. Each number after sample means the pooled IEX fractionated number. For O-Pec, fraction 1 was lost. Values are from one replicates. Coefficient of variation ranged from 1.21 to 5.43% for O-Pec, B-Pec and U-Pec.

Table 3. Texture profile analysis of pectin gels in the presence of 35 mM $CaCl_2$ at 2% pectin solution.

	Hardness (N)	Cohesiveness	Springiness (S)	Gumminess (N)	Chewiness (N·S)
B-Pec	0.80 [a]	0.67 [a]	1.52 [a]	0.53 [a]	0.81 [a]
B-Pec3	0.16 [b]	0.45 [ab]	1.76 [ab]	0.08[ab]	0.17[ab]
U-Pec	0.88 [a]	0.65 [a]	2.07 [a]	0.57[a]	1.20 [a]
U-Pec3	0.24 [b]	0.19 [b]	1.40 [b]	0.05 [b]	0.06 [b]
U-Pec4	0.19 [b]	0.27 [b]	1.68 [b]	0.05 [b]	0.09 [b]

[a] Mean values with different superscript in the same column are not significantly different at $p < 0.05$.

(B-Pec): SP-unbound and HP- bound PME modified pectin. (U-Pec): SP-unbound and HP-unbound PME modified pectin. Each number after sample means the pooled IEX fractionated number. O-Pec and other fractionated pectins did not gel in the presence of 35mM $CaCl_2$.

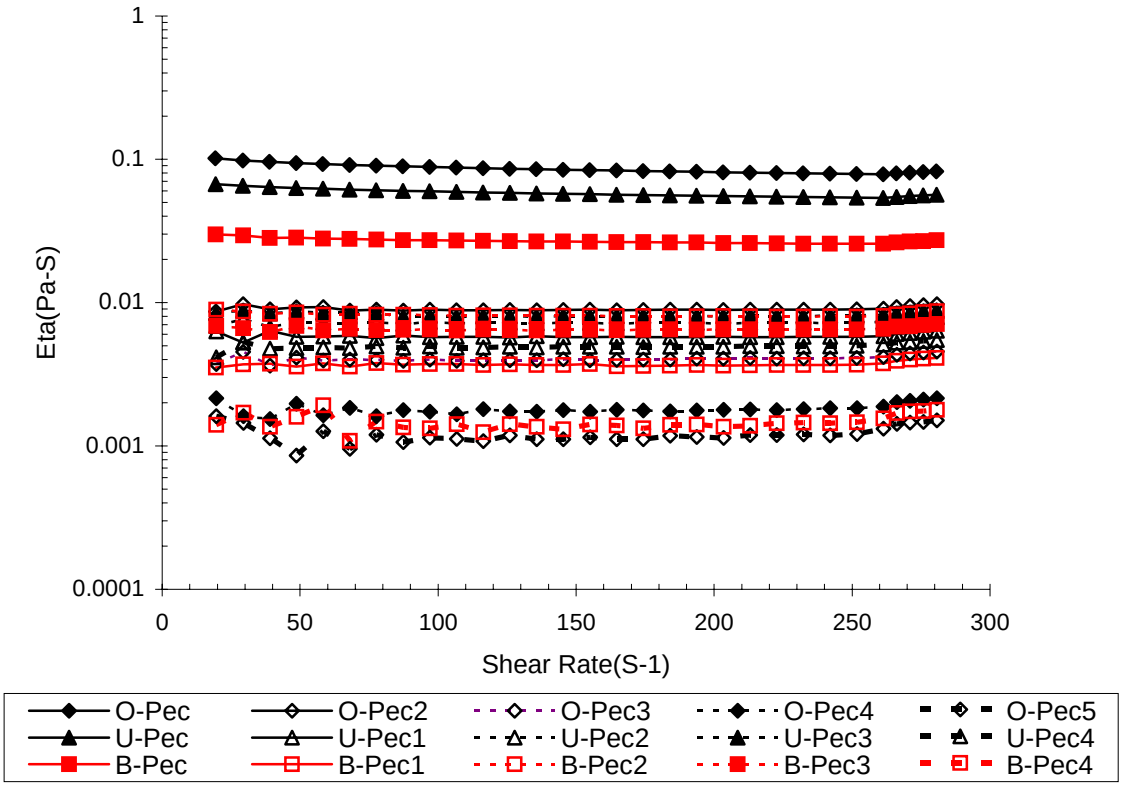

Figure 1. Plot of viscosity of 2% O-Pec, B-Pec and U-Pec without $CaCl_2$ in the range of shear rate between 10 and 280 s^{-1} at 20ºC.

(O-Pec): original pectin. (B-Pec): SP-unbound and HP- bound PME modified pectin. (U-Pec): SP-unbound and HP unbound PME modified pectin. Each number after sample means the pooled IEX fractionated number.

Table 4. The storage (G') and loss (G") modules of 2 % O-Pec, B-Pec, and U-Pec dispersion in the presence of 35 mM $CaCl_2$ at 1 Pa stress.

Sample	G' (Pa), 0.4 Hz	G" (Pa), 0.4 Hz	G' (Pa), 4 Hz	G" (Pa), 4 Hz
O-Pec	8.50	1.60	13.72	5.89
O-Pec2	0.02	0.02	1.08	-0.34
O-Pec3	0.01	0.01	1.40	-0.51
O-Pec4	0.01	0.01	1.33	-0.36
O-Pec5	0.06	0.09	1.77	-0.35
B-Pec	218.70	11.04	263.70	13.57
B-Pec1	0.16	0.15	1.15	0.08
B-Pec2	26.34	1.37	21.92	2.32
B-Pec3	108.4	4.81	124.1	5.57
B-Pec4	0.02	0.03	1.05	-0.15
U-Pec	502.50	22.55	585.60	29.22
U-Pec1	0.98	0.19	1.81	0.05
U-Pec2	27.42	1.22	24.70	1.75
U-Pec3	66.39	3.03	58.70	4.01
U-Pec4	69.49	2.50	91.81	2.73

(O-Pec): original pectin. (B-Pec): SP-unbound and HP bound PME modified pectin. (U-Pec): SP-unbound and HP unbound PME modified pectin. Each number after sample means the pooled IEX fractionated number.

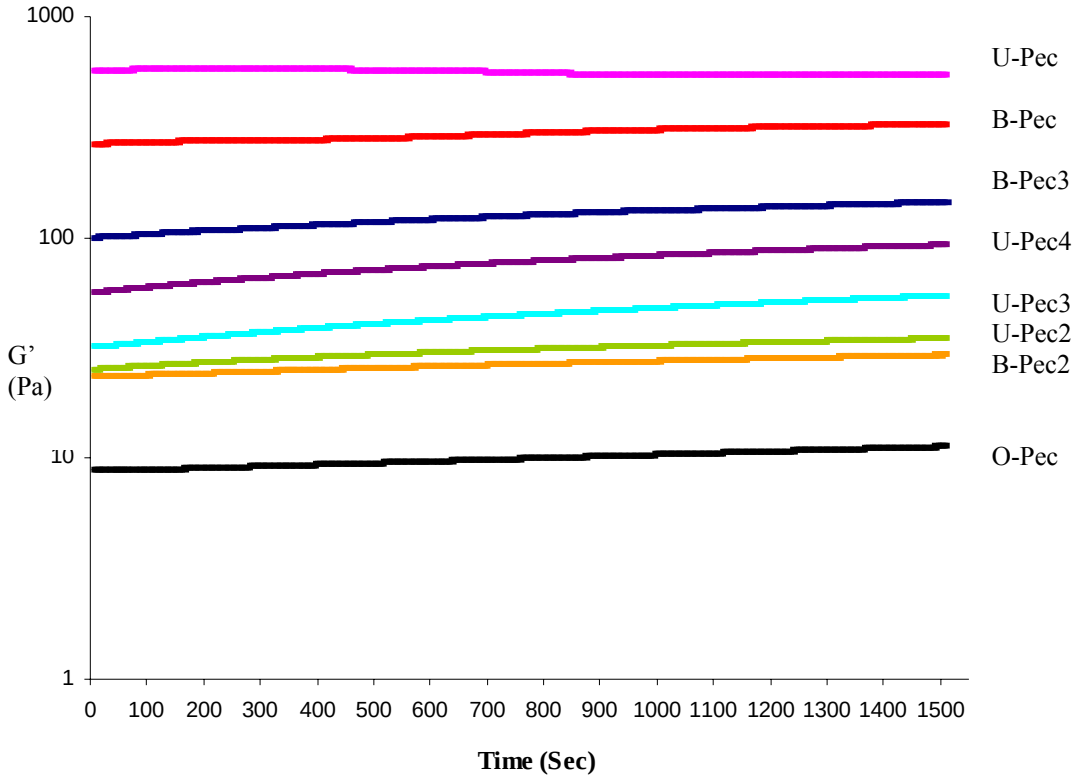

Figure 2. Storage modulus (G') as a function of time for 2% unfractionated and fractionated O-Pec, B-Pec, and U-Pec treated with at 1 Pa and 1 Hz.

(O-Pec): original pectin. (B-Pec): SP-unbound and HP bound PME modified pectin. (U-Pec): SP-unbound and HP unbound PME modified pectin. Each number after sample means the pooled IEX fractionated number.

CHAPTER 5

CHARGE DOMAIN OF MODIFIED PECTINS INFLUENCE INTERACTION WITH ACIDIFIED CASEINS [3]

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Abstract

The objective of the study was to investigate the interaction of the casein fractions with charge modified pectins (B-Pec and U-Pec) in acidified dispersion systems. Casein fractions in acetate buffer, pH 3.8 made a slight cloudy dispersion with precipitate, except κ-casein. A 1:10 mixture of pectins and caseins made dispersions with more precipitation. U-Pec led to more precipitation than O-Pec or B-Pec. Pellets had more protein than supernates from all samples except κ-casein dispersions. Caseins with pectin had larger particle size, especially U-Pec and B-Pec, than caseins without pectin. All casein dispersions without pectin showed positive or negative ζ-potential at pH 3.8 in the absence or presence of pectin, respectively. B-Pec and U-Pec added milk dispersions showed more negative ζ- potential than O-Pec. In non-fat milk, $\alpha S_{1,2}$ - and κ-casein, B-Pec was more effective at enhancing a negative ζ-potential than U-Pec. However, U-Pec enhanced negative ζ-potential of β-casein more than B-Pec. There was no significant difference in flow behavior (n) among non-fat milk samples regardless of pectins added. In contrast, modified pectin significantly decreased n and showed non-Newtonian behavior in pectin added κ-casein. Analysis by microscopy of non-fat milk and κ-casein showed that sizes and shapes were changed by addition of O-Pec and U-Pec.

Key words: Modified pectin, casein fractions, particle size, ζ- potential, viscosity, microscopy

Introduction

Acid milk drinks such as fruit milk drinks, yogurt drinks, soymilk, whey drinks etc. are composed of an acid dairy phase (fermented base) or a neutral base (milk) with an added acidic medium (fruit phase), sugar, and stabilizer (Laurent and Boulenguer, 2003). High methoxyl pectin is usually an effective stabilizer of acidic beverages to prevent sediment formation (Fleer and others, 1984; Pereyra and others, 1997; Maroziene and De Kruif, 2000). Namely, pectin is adsorbed onto the surface of casein micelles by electrostatic attraction, and the negatively charged pectin-casein complex is dispersed by electrostatic repulsion (Fleer and others, 1984; Parker and others 1994; Kratchenko and others 1995; Maroziene and De Kruif, 2000; Tuiner and others, 2002).

The interaction between milk protein and pectin at low pH in liquid or emulsion systems has been previously described by several groups. Whey protein with pectin had greater solubility and emulsifying properties at pH 4.6 compared to control whey protein (Mishra and others 2001). Pectin led to fine and stable emulsions similarly to gum arabic which is the most commonly recognized hydrocolloid emulsifier, but it can be used at lower dosage, because of the more extended conformation of the pectin molecules (Dickinson, 2003; Leroux and others 2003). Some studies reported the different efficiency to interact with milk protein depending on high methoxyl pectin (HMP) or low methoxyl pectin (LMP). The greater stabilizing effectiveness of HMP than LMP was attributed to the balance of interaction with the caseins. Anchoring and interaction of segments of HMP with solvent was contrasted to excess interaction of LMP with casein (Pereyra and others

1997). HMP is also effective under low pH conditions in stabilizing casein-coated emulsion drops. The pH required for effective stabilization of casein dispersion is in the range pH 3.7 – 4.2 where the casein and pectin have optimum opposite net charges (Dickinson, 1998). In acidified milk with sufficient pectin levels, the average size of the casein particle decreased to $< 1\mu m$, size distribution is more uniform, and the flow behavior is more Newtonian (Parker and others, 1994; Kravtchenko and others, 1995). There are few studies on protein-pectin interactions for individual caseins, as known for κ-carrageenan/casein. At pH values greater than the isoelectric precipitation of protein (pI_{pro}), the effect of HMP on the properties of casein-stabilized emulsions showed substantial differences in the protein-polysaccharide interactions for two major caseins, α -and β-Casein. The 60-fold higher viscosity of α-casein /pectin than β-casein/pectin under these conditions, pH 5.5 was due to the stronger attractive interaction in α-casein /pectin (Dickinson, 1998; Dickinson and others, 1998).

Caseins are the major milk proteins and are primarily composed αS_1, αS_2, β and κ-casein in the approximate proportion of 38, 10, 36 and 13 % (Davies and Law, 1980; Sood and others, 1992). The caseins are amphiphilic in nature arising from separation between hydrophobic and negatively charged regions along the peptide chain, resulting in self-assembly into submicelles (Swaisgood, 1997; Marchesseau and others, 2002). κ-casein is thought to coat the hydrophobic core of the submicelle and is important in casein/ polysaccharide interactions due to having a positively charged region available for electrostatic bonding (Langerendorf and others, 1999).

Pectin has a complex structure of α-(1-4)-D-galacturonic acid, partially esterified, L-rhamnose inserts into the backbone, and neutral sugar substitution (Aspinall, 1980; De Silva and others 1995). Pectin can be deesterified with plant pectinmethylesterase (PME) which facilitates $CaCl_2$ cross linking of pectin chains without sugar (Hotchkiss and others, 2002; Kim and others 2004). Laurent and Boulenguer (2003) found the calcium sensitive pectin is a more efficient stabilizer than the non-calcium sensitive pectin in acid dairy drinks. It may be caused by the total charge and distribution of charge that influences the calcium sensitivity of pectins (Kim and others, 2004). Kazmierski and others (2003) reported that mHMP that is modified using plant PME was more reactive with β-lactoglobulin than HMP, which was attributed to the blockwise charge distribution of mHMP. In this study, we compare the interaction of individual caseins with pectins of unique charge properties. Although several studies of the interaction between pectin and caseins in liquid or emulsion systems exist, there is little research on interaction of individual casein fractions with charge modified pectins in dispersed systems. Such knowledge is helpful in development of tailored pectins for stabilization of milk proteins in specific systems.

Materials and Methods

Materials

Commercial, unstandardized, high methoxyl pectin (citrus pectin type 104, 73 % degree of esterification (%DE), CP Kelco, Lille Skensved, Denmark) was modified by Valencia PME as described by Kim and others (2004) to prepare unmodified (O-Pec) or

charge modified (B-Pec 63 % DE, U-Pec 61 %DE). U-Pec or B-Pec was de-esterified by PME fractions that varied in binding affinity for SP-sepharose and heparin columns.

Caseins were isolated using the method described by Hollar and others (1991). Low heat, non-fat dry milk (Dietrich's Milk Products Inc., Reading, PA) was dissolved as 15% solids in 2L of deionized water and heated to 30ºC. Milk pH was adjusted to 4.6 with 1N HCl. Whey was drained twice with cheesecloth and the casein precipitate was washed with deionized water. The precipitate was resuspended in distilled water and pH was adjusted to 6.7 with 1N NaOH. Casein was freeze-dried and stored at –20ºC. Casein fractions, $\alpha S_{1,2}$-casein and β-casein, were separated by an Akta purifier system consisting of a P-900 HPLC pump, a UV-900 detector measuring at 280nm (Kazmierski, 2002). κ-Casein was used from commercial bovine κ-casein (Sigma Chemical Co., St. Louis, MO. C0406-1G, lyophilized powder, minimum 80% κ-casein).

Mixture of Milk Protein and Pectin

A 1% dispersion of milk proteins were prepared in sodium acetate buffer (50 mM, pH 3.8) and incubated for 2 hour at 20°C. O-Pec, U-Pec or B-Pec was added to achieve a ratio of 1:10 pectin: protein.

Soluble Protein and Pectin

A mixture of milk protein and pectin in eppendorf tubes was centrifuged in a microfuge (Microfuge 5421, Brinkmann Ins. NY) for 5 min. The pellet and supernate were carefully separated. The pellet was resuspended with 1ml sodium acetate buffer (50mM, pH 6.0) for 10 min. The protein content of pellet and supernate was measured using the

bicinchoninic acid (BCA) protein assay (Smith and others 1985) after diluted by instructions provided by manufacturer (Pierce Biotechnology, Rockford, Il). Galacturonic acid was estimated using colorimetric assay (Blumenkrantz and Asboe-Hansen 1973).

Particle Size Distribution

The particle size distribution was determined by laser diffraction using a Mastersizer S, (Malvern Instruments, Southborough, MA) as described by (Ackerley and Wicker, 2003). Size distributions of 1% pectin/protein (1:10) dispersions were calculated on the volume fractions against particle size and the weight-average size were expressed as $d_{3,2}=(\Sigma n_i d_i^3/\Sigma\ n_i d_i^2)$ and $d_{4,3}=(\Sigma n_i d_i^4/\Sigma\ n_i d_i^3)$, where n_i is the number of particles of diameter d_i.

Zeta (ζ)- Potential and Mobility

Dispersions of 0.4% (w/w) pectin/protein (1:10) dispersions were adjusted to pH values between 3.0 and 7.0 by HCl or NaOH. Measurement of ζ-potential was performed with a Particle Size Analyzer adding the BI-Zeta option (90 Plus, Brookhaven Inst., Holtsville, NY) with a 50 mW diode laser (90 angle) and a BI-9000AT correlator. Experiments were carried out at 25°C with the laser beam operation at 659.0 nm and 1.330 as the refractive index. The measurements were carried out in triplicate of 2 min each and 5sec between each run. The ζ-potential was determined subsequently after the particle size determination for the same sample of pectin solution.

Viscosity

The viscosity of casein solution with modified pectin was determined using a Controlled Stress Dynamic Rheometer™ (Rheometrics, Piscataway, New Jersey, U.S.A.) equipped with a cone and plate device (60 mm diameter, 0.0385°, 0.5 mm gap). Flow curves of 1.3 ml of 1% pectin/protein (1:10) dispersions increased by shear rate (10 ~50 s^{-1}) at 20°C. Shear rate against shear stress data were fit using the power law ($\tau=k\cdot\gamma^n$), and analyzed on the flow behavior, n and consistency index, k.

Microstructural Studies

Light microscopy was performed using a Carl Zeiss Axiomat microscope (Carl Zeiss Photomicroscope III, New York, NY,USA), at 20X lens. Samples of milk treated with 50 mM acetate buffer, pH 3.8 were placed on a hollow slide and covered with a cover slide without staining. Images acquisition and analysis were managed using Zeiss software (Version 2.5).

Statistical Analysis

Results from triplicate assays were analyzed by the analysis of variance (AVOVA) using the SAS program (version 8.0, Cary, NC..).The difference of means among the samples were resolved by the least significant difference (LSD) at significance level $p < 0.05$.

Results and Discussion

Visual Appearance

The visual appearance of dispersions varied with the type of milk protein and pectin. Non-fat milk, casein, $\alpha S_{1,2}$ - and β-casein fractions made slightly cloudy dispersions in

acetate buffer, pH 3.8. Only the κ-casein dispersion gave a clear appearance at pH 3.8. The appearance of the dispersions after 2 hour at 20°C is depicted in Figure 1. Addition of pectin to the milk fractions had unique effect and ranged from increasing the extent of sedimentation (Fig. 1a, c, d, e) to increasing the opalescent appearance (Fig 1b). Non-fat milk at pH 3.8 had a greater precipitate, in the presence of O-Pec, B-Pec, or U-Pec. On the other hand, the opalescent appearance of casein at pH 3.8 increased in the presence of O-Pec, B-Pec, or U-Pec with the latter modified showing the greatest effect. The different effect between non-fat milk and casein in the presence of pectin suggests that non-casein components in non-fat milk contribute to greater instability. Of the fractionated caseins, O-Pec, B-Pec, or U-Pec had the least effect on appearance of $\alpha S_{1,2}$-casein. The dispersion was slightly more opalescent and had slightly larger precipitate, regardless of type of pectin. The opalescence of β-casein was minimally affected by pectins, but the size of the precipitate increased with pectin, especially U-Pec. Although κ-casein had no precipitate initially, addition of pectin resulted in sedimentation, and U-Pec resulted in the greatest precipitate. U-Pec had the greatest effect on dispersions than B-Pec or O-Pec.

Boulenguer and Laurent (2003) suggested that the increasing sediment was due to an increase in the thickness of the pectin layer. In a study of calcium sensitive (CS) and non-calcium sensitive (NCS) as stabilizer in acid dairy drinks, Laurent and Boulenguer (2003) found the CS pectin yields a greater sediment weight than the NCS pectins. Pereyra and others (1997) also reported that HMP (less calcium sensitive) had less precipitate than LMP (more calcium sensitive) in acidified caseinate dispersions. In this study, U-Pec is

more calcium sensitive than B-Pec and O-Pec (Kim and others, 2004). Thus the greater sedimentation in the presence of U-Pec may be due to more binding with the cationic protein.

Protein and Pectin Content in Mixtures

Table 1 shows the content of protein and pectin in pellet and supernate after centrifugation, respectively. In protein content, pellet had more protein than supernate for most samples. Only the κ-casein dispersion had more protein in supernate, 8.52 mg/ml than in pellet, 1.33 mg/ml. Addition of pectin to the milk proteins led increased protein content in pellet except non-fat milk. B-Pec and U-Pec increased protein content in pellet more than O-Pec, especially for β- or κ- casein. In κ- Casein, the amount of protein in pellet increased in the order of only κ- casein, O-Pec added, B-Pec added, and U-Pec added κ- casein.

In pectin content, the tendency was similar to that of protein when pectin was added, but the difference among casein fractions was greater. Namely, in β- or κ- casein, B-Pec or U-Pec addition led to more pectin in pellet than O-Pec, but in $\alpha S_{1,2}$ –casein, O-Pec made more pectin in pellet than B-Pec or U-Pec. There was no difference regardless of the type of pectin in non-fat milk. The small amount of uronic acid detected in non-fat milk, no pectin dispersions is likely due to lactose interference with the pectin assay.

It is suggested that protein associated with pectin played a key role in the stabilization of the emulsion (Leroux and others, 2003). The high amount of precipitated protein suggested that casein could be involved in the interaction with pectin. In the other

words, the fraction which became associated with the oil contained more protein than the fraction in the aqueous phase. Girard and others (2002) found that β-lg complexed with LMP around 96%, whereas only 78% complexed with HMP at pH 4.5. This is in the agreement with this result. Namely, O-Pec (72% DE) treated dispersions normally had less protein or pectin content than B-Pec (63 %DE) or U-Pec (60 %DE).

Particle size

Particle size distributions were calculated on the weight-average size (Table 2). The average dispersion particle size was found to be larger in pectin added dispersion than in dispersion without pectin except $\alpha S_{1,2}$ -casein. In non-fat milk and κ-casein, all sample was significantly different $D_{4,3}$ and B-Pec or U-Pec added dispersions showed larger particle size than O-Pec added dispersions ($p < 0.05$). κ-Casein particle size could not be measured due to small size. However, in the presence of pectins, there was a dramatic increase in $D_{4,3}$ values: KCO (135.2μm), KCB (215.3μm), and KCU (258.3μm). The particle size of β-casein also increased following the same tendency of non-fat milk dispersion and κ-casein, but U-Pec added dispersion had only significantly different particle size comparing O-Pec, B-Pec – added or no added dispersion. However, $\alpha S_{1,2}$ -casein dispersions showed there was no significant change in particle size regardless of type of pectin and decreased compared to no pectin. At the volume to surface average diameter, $D_{3,2}$, there were no significant difference ($p >0.05$) among samples even though there are increase after pectin addtion except κ-casein dispersions. In protein only dispersions, there was a high standard deviation among the measurements. It may probably be caused by coagulated casein

dispersion at pH 3.8 and the dispersion leads to a greater heterogeneous distribution of size (Data was not shown).

The particle size against size volume fractions for individual casein fractions give a better understanding of the change on particle size after addition of pectins (Figure 2-1, 2-2, and 2-3). Non-fat milk dispersions showed different distribution from other casein fractions (Figure 2-1). After adding pectin, non-fat milk dispersion showed two main particle populations. Adding pectin led lower $D_{3,2}$ values and higher $D_{4,3}$ values in the order of O-Pec, B-Pec and U-Pec. In casein (Figure 2-2), pectin addition to dispersions reduced smaller particle size. There was little difference in similar particle size distribution among O-Pec, B-Pec and U-Pec dispersions. In contrast, in β- and κ-casein (Figure 2-3a, b) dispersions with pectin had larger particle size than dispersions without pectin. In $\alpha S_{1,2}$-casein, all dispersions had similar particle size regardless of presence of pectin but distribution was variable. Based on the results, we can assume κ-casein may be the most effective casein system for comparing the interaction with other polysaccharide, especially U-Pec.

Creamer and Berry (1975) provided evidence that the submicelles were not homogenous with respect to size. Dickinson and others (1998) studied stability of emulsions with the same pectin content that were made with $\alpha S_{1,2}$-casein, β-casein, or sodium caseinate. Those three emulsions showed a similar average droplet size and stability properties, except in the case of $\alpha S_{1,2}$-casein based emulsions at ionic strength > 0.1 M. For $\alpha S_{1,2}$-casein, it is not influenced by pectin addition because it has only

extensive flocculation. Schmidt and Buchhem (1975) reported the size distributions in solution of $\alpha S_{1,2}$-casein differed considerably from those of β-, and κ-casein fractions. $\alpha S_{1,2}$-casein showed a steady decrease in particle number with increasing diameter, whereas β-, and κ-casein exhibited a maximum in their particle size distributions. This difference may be explained on the basis of the known association behavior of the individual casein components. αS_1-Casein is known to undergo a series of consecutive association steps of which the equilibrium constants are of the same order of magnitude (Schmidit, 1970). This results in broad distribution of monomers and polymers in which the number of polymer molecules decrease with the degree of polymerization and thus with their size. In contrast to the indefinite association of $\alpha S_{1,2}$-casein, β-casein shows a discrete, micellar type of association (Schmidt and Payens, 1972). From sedimentation behavior and molecular weight determinations, it has been concluded that in solutions of β-casein only large polymers with a narrow size distribution occur in addition to monomers. The association of κ-casein in an ultracentrifugal filed was similar to that of β-casein except that the association equilibrium had strongly shifted to the polymer side (Schmidt and Buchhem, 1975; Vreeman 1979)

Surface Charge by Zeta (ζ)-Potential

Table 3 presents the ζ-potential and mobility of 1% dispersion of non-fat milk and casein fractions with pectins (1:10) in acetate buffer, pH 3.8. All milk dispersions without pectin showed positive ζ-potential. The range of ζ-potential in individual casein fractions was between 12.84 and 22.13 mV. In the presence of pectin, negative ζ-potential was

observed regardless of the type of pectin in all milk dispersions. Usually, B-Pec or U-Pec added dispersions led to more negative ζ-potential than O-Pec added dispersions. For non-fat milk, $\alpha S_{1,2}$ -and κ- caseins, B-Pec added dispersions had higher negative ζ-potential than U-Pec added dispersions. U-Pec added dispersions had higher negative ζ-potential than B-Pec added dispersions for β-casein. In casein dispersions, there was no difference in ζ-potential between B-Pec and U-Pec.

The stability of the casein micelles at milk pH has been attributed in part to the net negative charge on the surface of the micelles (Anema and Klostermeyer, 1996). At neutral pH, ζ-potential for casein micelles were reported around –18 mV~ -20 mV because carboxyl groups partly became negatively charged ($-COO^-$) and partly because neutral ($-NH_2$). Between pH 5.8 and 5.5, ζ-potential of milk decreased and particle size increased from 180 to 1300 nm. Below the isoelectric point of the casein protein (~ pH 5.0), milk dispersions showed positive ζ-potential because the amino groups are positively charged ($-NH_3^+$), and the carboxyl groups are neutral (-COOH) (Dalgleish, 1984; Laurent and Boulenguer, 2003; Theresa and others, 1996). As pectin is acidic polysaccharides having galacturonic acid as a component sugar, ζ-potential is negative at pH 3.8. Therefore, adding pectin caused increase in negative charge or decrease in positive charge on the casein dispersions (Nakamura and others, 2003). Under acidic condition that is below pI_{pro}, pectin is adsorbed onto the surface of casein micelles by electrostatic attraction, and then the negatively charged pectin-casein complex is dispersed by electrostatic repulsion (Fleer and other, 1984; Maroziene and De Kruif, 2000). As above results, modified pectin added

casein dispersions greater a negative ζ-potential than unmodified pectin added one. It may be attributed by different charge and charge density through Valencia PME modification. At previous study (Kim and others, 2004), we showed that Valencia PME modification led to more charge density through the blockwise structure by slight decreasing DE in HMP. In addition, B-Pec and U-Pec had more calcium sensitive and charge density than O-Pec. In addition, individual casein fractions also has different chemical structure, thus it may interact with pectin in different way.

Viscosity of Casein and Pectin Dispersion

Flow behavior (n) and consistency index (k) of 1% milk protein added O-Pec, B-Pec, or U-Pec are presented at Table 3. Normally, milk dispersion shows Newtonian flow behavior as following from non-fat milk at this study. No matter which modified pectin add, there was no significant difference in n and k value among non-fat milk dispersion ($p<0.05$). In contrast, for κ-casein dispersions, modified pectins in dispersions significantly decreased n values and showed non-Newtonian behavior, KC (0.94), KCU (0.79) and KCO (0.66). k value interpreted as relative thickness values in fluid is was also significantly difference in depending on modification of pectin. However, there was no difference in viscosity among milk dispersions. During the range of shear rate between 0 and 30 S^{-1}, there was not much change in viscosity. κ-Casein dispersions had 2-fold higher than non-fat milk dispersion. Viscosity was around 0.002 Pa^{-S} (NF and NFU) and 0.004 (NFO) at shear rate 30 S^{-1}.

Normal milk behaves as a Newtonian liquid. There is a transition from Newtonian to non-Newtonian behavior as like the concentration is increased. It is due to the removal of water causing an increase in volume fraction of dispersed particles and an increase the micelle-micelle interactions as the distance between the micelles becomes smaller (Velez-Ruitz and Barbosa-Canovas, 2000; Walstra and Jenness, 1984). Pereyra and other (1997) reported the dispersion of LMP/casein or HMP/casein was less Newtonian and higher k value than LMP or HMP. Maroziene and De Kruif (2000) showed the addition of pectin to skim milk hardly increased the viscosity at pH 6.7. Glahn (1982) reported that viscosity of acidified milks containing low concentrations of pectin had an initial sharp increase and followed by a sharp decline at higher pectin levels. However, Laurent and Boulenguer (2003) reported that the viscosity observed at high pectin concentrations (2500ppm) was much higher for calcium sensitive (CS) pectin than non-calcium sensitive pectin for non-fat milk because of the presence of a network and depletion phenomena for the CS pectin. This difference among studies may be from several factors like a concentration of pectin, mixing ratio, or measuring factors.

Optical Microstructure

Some typical examples of particles in dispersion of non-fat milk and κ-casein are depicted at Figure 3. Analysis by optical microscopy of the aggregates of casein micelles with pectin showed that their sizes and shapes changed with addition of pectin. In non-fat milk dispersion at pH3.8, the transparent and opaque granules were still detectable and heterogeneous with respect to size. These are typical and basic constituents of these

morphological configurations of the casein (Calapal, 1968). The photograph of non-fat milk shows discrete, clearly defined particles that appear firm and spherical, but in disperion with pectin, the micelle appears to have adhered to one another and aggregates. For the κ-casein dispersion, small and almost spherical particles were distributed evenly. However, after addition of pectins, bigger size of aggregates appeared and their shapes were different. κ-Casein/pectin aggregates were more irregular than non-fat milk/pectin aggregates. For further finding the way to interaction between casein proteins and pectins, the sample was stained (not presented here). On the photograph of the stained aggregates, mainly protein part was in the center and pectin part seem be in the junction of two protein parts, like a cement. However, further study need to distinguish the junction and the respective location of each macromolecule in the complexes.

Conclusions

Based on the comparison with dispersion systems of individual casein fractions, $\alpha S_{1,2}$-, β-, and κ-casein, in the presence of charge modified pectins, each casein fractions interacted uniquely depending on modified pectins. Especially, κ-casein is distinguished from other casein fractions. β-casein seems to interact with $\alpha S_{1,2}$-casein and modified pectins to stabilize dispersion. For the modified pectins' availability on acidic milk system, we compared the unmodified and two modified pectins which have the blockwise charge distribution through the plant PME modification. Between two modified pectins, more calcium sensitivity modified pectin (U-Pec) seems more reactive than the less calcium sensitive pectin (B-Pec) in terms of interaction with milk protein. Ultimately, interaction of

individual casein fractions with charge modified pectins in dispersed systems gives an idea for the development of tailored pectins for stabilization of milk proteins in acidified dispersions.

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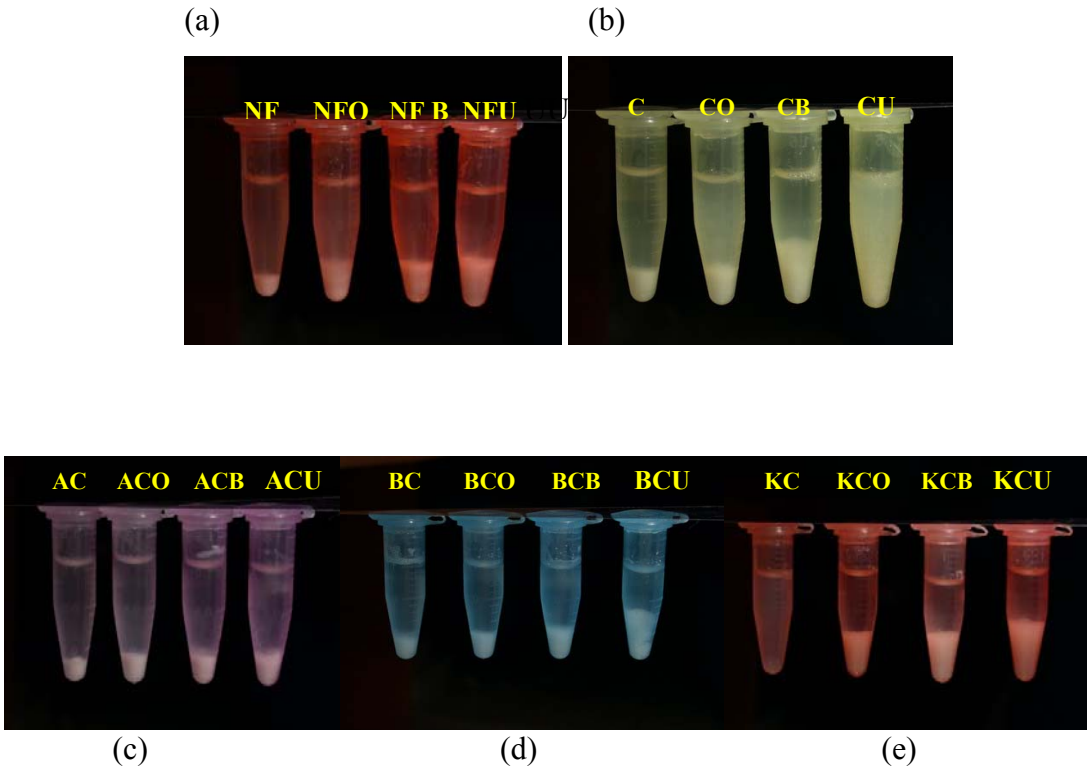


Figure 1. Photograph of 1% milk dispersion with pectins (10:1) following sit at 20°C for 2hour.

(a) NF: non-fat milk, NFO: NF in O-Pec, NFB: NF in B-Pec, NFU: NF in U-Pec (b) C : Casein, CO: C in O-Pec, CB: C in B-Pec, CU: C in U-Pec (c) AC : $\alpha S_{1,2}$-Casein, ACO: AC in O-Pec, ACB: AC in B-Pec, ACU: AC in U-Pec (d) BC : β-Casein, BCO: BC in O-Pec, BCB: BC in B-Pec, BCU: BC in U-Pec (e) KC : κ-Casein, KCO: KC in O-Pec, CB: KC in B-Pec, KCU: KC in U-Pec

Table 1. Protein and pectin content in pellet and supernate of 1% dispersion milk protein with pectins (10:1) in acetate buffer, pH 3.8.

Sample		Protein Content (mg/ml)		Pectin Content (mg/ml)	
		Supernate	Pellet	Supernate	Pellet
Non-Fat milk	NF	3.38 ± 0.15	5.33 ± 0.15	0.19 ± 0.07	0.19 ± 0.08
	NFO	3.18 ± 0.24	4.63 ± 0.12	0.48 ± 0.03	0.46 ± 0.04
	NFB	3.56 ± 0.05	5.07 ± 0.03	0.21 ± 0.05	0.63 ± 0.05
	NFU	3.41 ± 0.10	5.74 ± 0.39	0.19 ± 0.06	0.54 ± 0.09
$\alpha S_{1,2}$ -Casein	AC	1.63 ± 0.15	8.72 ± 0.25	0.00 ± 0.01	0.01 ± 0.01
	ACO	0.72 ± 0.28	10.35 ± 0.31	0.17 ± 0.02	0.99 ± 0.08
	ACB	1.75 ± 0.48	8.19 ± 0.16	0.18 ± 0.01	0.93 ± 0.04
	ACU	1.14 ± 0.18	9.32 ± 0.25	0.15 ± 0.01	0.74 ± 0.04
β-Casein	BC	3.45 ± 0.31	5.30 ± 0.42	0.01 ± 0.01	0.00 ± 0.01
	BCO	3.30 ± 0.26	4.75 ± 0.22	0.11 ± 0.03	0.55 ± 0.08
	BCB	2.43 ± 0.21	8.40 ± 0.41	0.14 ± 0.03	0.67 ± 0.03
	BCU	2.65 ± 0.32	8.40 ± 0.08	0.14 ± 0.03	0.68 ± 0.10
κ-Casein	KC	8.52 ± 0.02	1.33 ± 0.15	0.01 ± 0.02	0.00 ± 0.00
	KCO	3.29 ± 0.27	8.12 ± 0.35	0.12 ± 0.04	0.45 ± 0.08
	KCB	1.47 ± 0.35	7.41 ± 0.30	0.11 ± 0.03	0.73 ± 0.07
	KCU	0.85 ± 0.13	9.89 ± 0.18	0.11 ± 0.03	0.85 ± 0.10

NF: non-fat milk, NFO: NF in O-Pec, NFB: NF in B-Pec, NFU: NF in U-Pec// AC : $\alpha S_{1,2}$ -Casein, ACO: AC in O-Pec, ACB: AC in B-Pec, ACU: AC in U-Pec // BC : β-Casein, BCO: BC in O-Pec, BCB: BC in B-Pec, BCU: BC in U-Pec// KC : κ-Casein, KCO: KC in O-Pec, CB: KC in B-Pec, KCU: KC in U-Pec

Table 2. The weight-average size of 1% dispersion milk protein with pectins (10:1) in acetate buffer, pH 3.8.

Sample		Weight Average Size	
		$D_{3,2}$ (μm)	$D_{4,3}$ (μm)
Non-fat milk	NF	7.74 [a] ± 0.11	25.8 [a] ± 1.63
	NFO	11.28 [a] ± 1.07	56.74 [b] ± 13.33
	NFB	15.27 [a] ± 0.50	102.4 [c] ± 14.13
	NFU	21.5 [a] ± 2.19	183.6 [d] ± 13.91
$\alpha S_{1,2}$ -Casein	AC	5.87 [a] ± 0.25	44.63 [a] ± 14.21
	ACO	6.51 [a] ± 0.20	24.18 [b] ± 2.99
	ACB	7.52 [a] ± 0.04	34.55 [b] ± 5.00
	ACU	7.78 [a] ± 0.35	33.68 [b] ± 1.22
β-Casein	BC	5.67 [a] ± 0.12	24.00 [a] ± 4.71
	BCO	13.37 [a] ± 1.15	29.12 [a] ± 1.11
	BCB	10.28 [a] ± 0.65	28.71 [a] ± 4.07
	BCU	14.71 [a] ± 1.43	43.23 [b] ± 6.23
κ-Casein	KC	ND	ND
	KCO	35.94 [a] ± 6.22	135.2 [a] ± 7.34
	KCB	39.13 [a] ± 7.13	215.3 [b] ± 43.93
	KCU	69.29 [b] ± 44.87	258.3 [c] ± 5.91

[a] Under same milk protein, means with the same superscript in a column are not significantly different at p = 0.05.
where n_i is the number of particles of diameter d_i.
ND: no determination

NF: non-fat milk, NFO: NF in O-Pec, NFB: NF in B-Pec, NFU: NF in U-Pec// AC : $\alpha S_{1,2}$ -Casein, ACO: AC in O-Pec, ACB: AC in B-Pec, ACU: AC in U-Pec // BC : β-Casein, BCO: BC in O-Pec, BCB: BC in B-Pec, BCU: BC in U-Pec// KC : κ-Casein, KCO: KC in O-Pec, CB: KC in B-Pec, KCU: KC in U-Pec

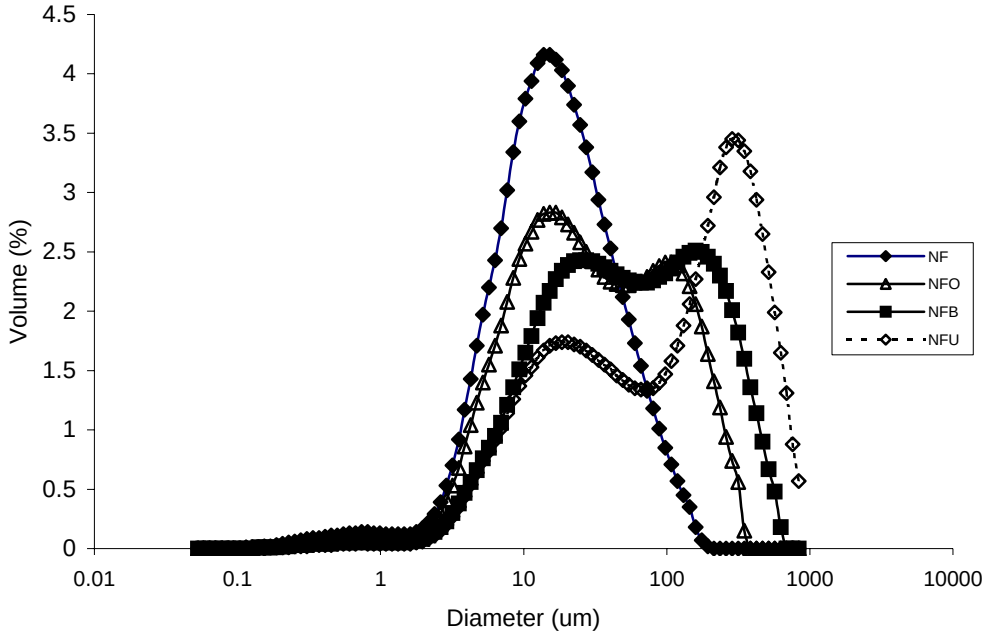


Figure 2-1. Particle size distribution of 1% dispersion of non-fat milk protein with pectins (10:1) in acetate buffer, pH 3.8. Percentage transmittance at 650nm values given in legend.

(NF): non-fat dry milk, (NFO):non-fat dry milk with O-Pec, (NFB):non-fat dry milk with B-Pec, (NFU):non-fat dry milk with U-Pec

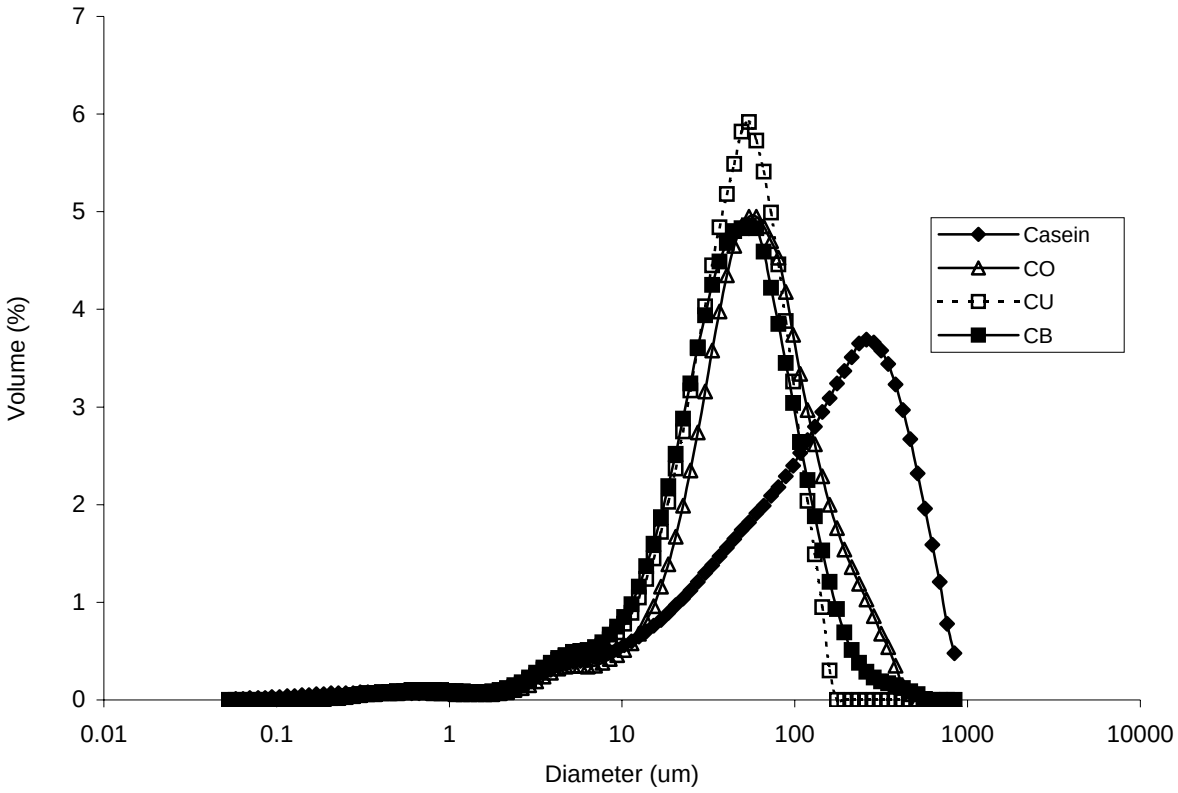


Figure 2-2. Particle size distribution of 1% dispersion of casein with pectins (10:1) in acetate buffer, pH 3.8. Percentage transmittance at 650nm values given in legend. (CO): casein with O-Pec, (CB): casein with B-Pec, (CU): casein with U-Pec

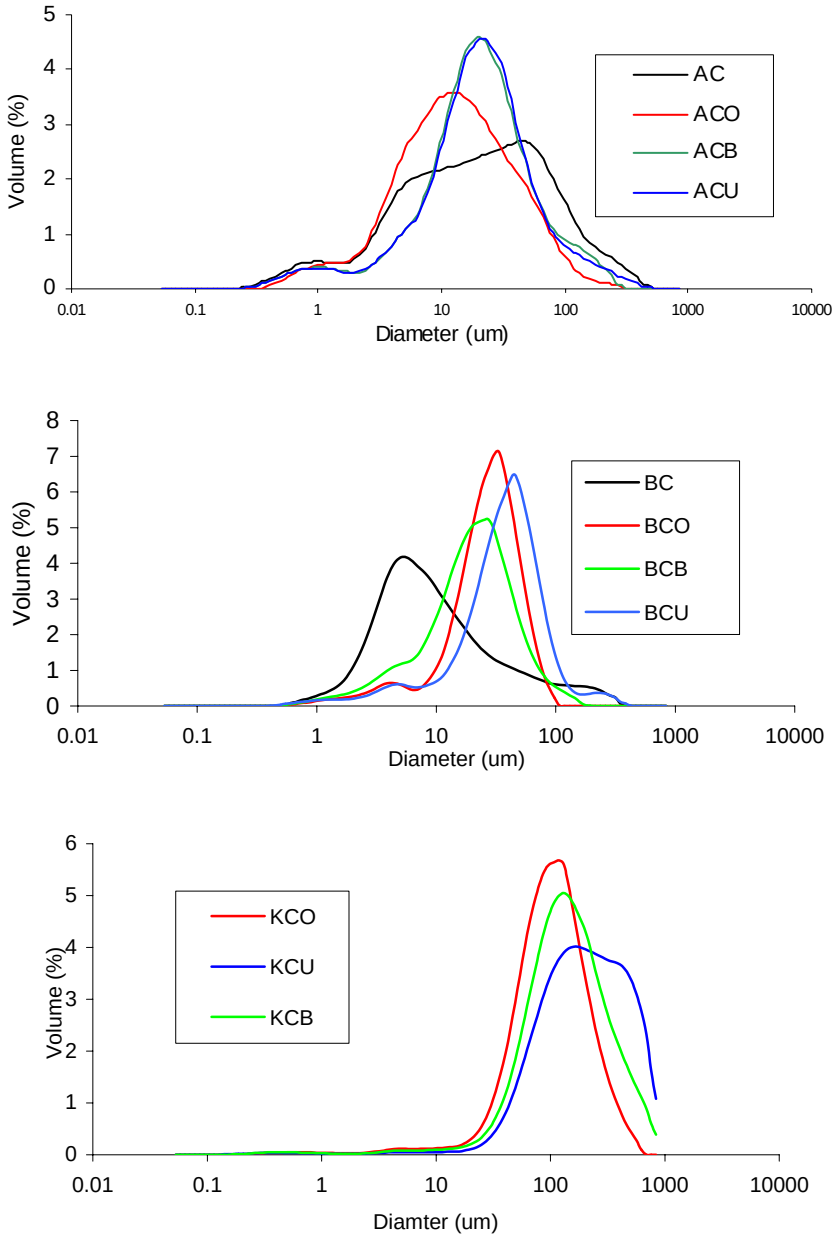


Figure 2-3. Particle size distribution of 1% dispersion of casein fractions with pectins (10:1) in acetate buffer, pH 3.8. Percentage transmittance at 650nm values given in legend.

(a): AC : $\alpha S_{1,2}$-Casein, ACO: AC in O-Pec, ACB: AC in B-Pec, ACU: AC in U-Pec (b): BC : β-Casein, BCO: BC in O-Pec, BCB: BC in B-Pec, BCU: BC in U-Pec (c): KC : κ-Casein, KCO: KC in O-Pec, CB: KC in B-Pec, KCU: KC in U-Pec. KC can't measure the particle size.

Table 3. Zeta- potential and mobility of 1% dispersion of casein fractions with pectins (10:1) in acetate buffer, pH 3.8.

Sample		Zeta-Potential (mV)	Mobility
Non-fat milk	NF	12.84 ± 2.98	1.00 ± 0.23
	NFO	- 7.23 ± 4.20	-0.56 ± 0.33
	NFB	-20.11 ± 3.20	-1.57 ± 0.25
	NFU	- 8.64 ± 3.01	-0.68 ± 0.24
Casein	C	17.44 ± 3.54	1.36 ± 0.28
	CO	-10.81 ± 3.00	-0.84 ± 0.23
	CB	-19.36 ± 2.37	-1.51 ± 0.19
	CU	-20.63 ± 1.46	-1.61 ± 0.11
$\alpha S_{1,2}$-Casein	AC	18.75 ± 4.70	1.47 ± 0.37
	ACO	- 8.78 ± 0.44	-0.69 ± 0.03
	ACB	-20.93 ± 6.15	-1.64 ± 0.48
	ACU	-14.25 ± 5.05	-1.11 ± 0.39
β-Casein	BC	15.09 ± 14.13	1.18 ± 1.10
	BCO	- 3.35 ± 3.19	-0.26 ± 0.25
	BCB	- 4.88 ± 3.27	-0.38 ± 0.26
	BCU	-21.84 ± 2.04	-1.71 ± 0.16
κ-Casein	KC	22.13 ± 0.69	1.73 ± 0.05
	KCO	4.44 ± 1.56	0.35 ± 0.12
	KCB	- 8.59 ± 3.86	-0.67 ± 0.30
	KCU	- 2.93 ± 0.94	-0.23 ± 0.07

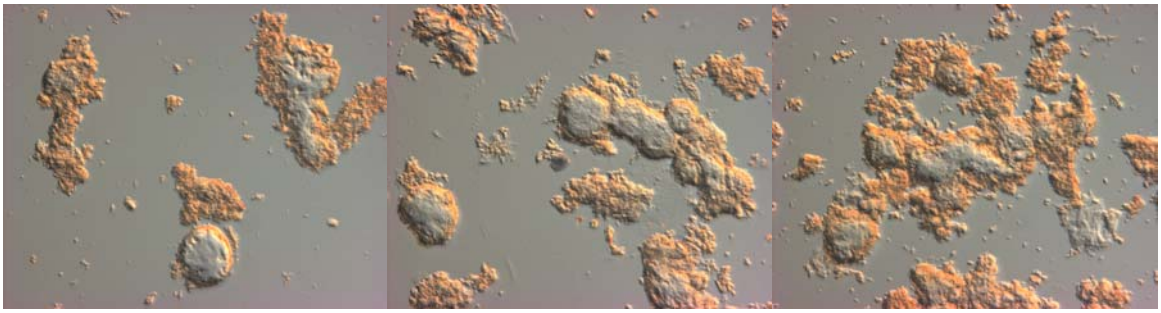
NF: non-fat milk, NFO: NF in O-Pec, NFB: NF in B-Pec, NFU: NF in U-Pec // C : Casein, CO: C in O-Pec, CB: C in B-Pec, CU: C in U-Pec // AC : $\alpha S_{1,2}$-Casein, ACO: AC in O-Pec, ACB: AC in B-Pec, ACU: AC in U-Pec // BC : β-Casein, BCO: BC in O-Pec, BCB: BC in B-Pec, BCU: BC in U-Pec // KC : κ-Casein, KCO: KC in O-Pec, CB: KC in B-Pec, KCU: KC in U-Pec.

Table 4. Viscosity Properties (n, Flow behavior and k, Consistency index) of 1% Milk Protein added 0.1% Original or Modified pectins.

Sample		n	k ($Pa \cdot S^n$)
Non-fat milk	NF	$1.057^{a} \pm 0.06$	$0.002^{a} \pm 0.000$
	NFO	$1.054^{a} \pm 0.08$	$0.003^{a} \pm 0.001$
	NFU	$1.042^{a} \pm 0.04$	$0.002^{a} \pm 0.000$
κ-Casein	KC	$0.937^{a} \pm 0.07$	$0.005^{a} \pm 0.004$
	KCO	$0.659^{b} \pm 0.03$	$0.017^{ab} \pm 0.005$
	KCU	$0.797^{c} \pm 0.03$	$0.024^{b} \pm 0.022$

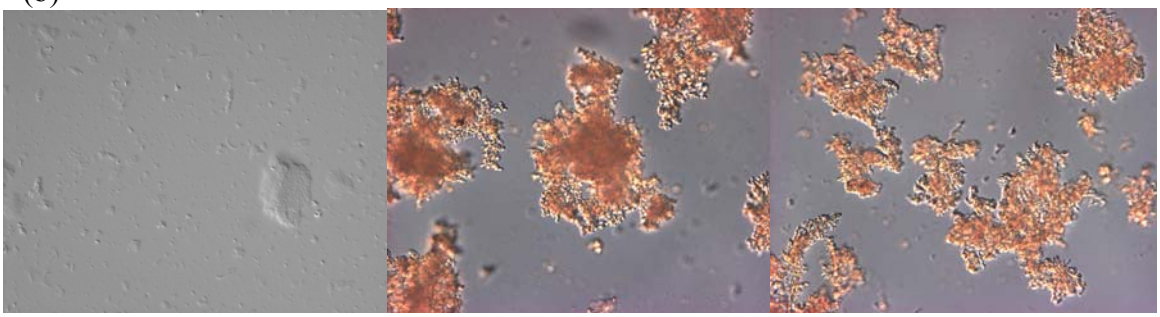
[a] Under same milk protein, means with the same superscript in a column are not significantly different at p = 0.05.
NF: non-fat milk, NFO:NF in O-Pec, NFU: NF in U-Pec
KC : κ-Casein, KCO: KC in O-Pec, KCU: KC in U-Pec.

(a)



NF NF+O-Pec NF+ U-Pec

(b)



KC KC+ O-Pec KC+U-Pec

Figure 3. Photomicrographs of non-fat milk protein and κ-Casein dispersions prepared with pectin in a light microscope at 20X.

(a) NF: non-fat milk, NFO:NF in O-Pec, NFU: NF in U-Pec (b) KC : κ-Casein, KCO: KC in O-Pec, KCU: KC in U-Pec

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CONCLUSIONS

The objective of this study was to de-esterify pectin by Valencia PME fractions (B-Pec containing the 36 and 13 kDa peptides and U-Pec containing the 36 and 27 kDa peptides), and characterize the resultant pectins for charge and charge distribution. Especially, the calcium sensitivity of modified pectins was investigated. Finally, the interaction of individual caseins with modified pectins of unique charge properties was compared.

Valencia PMEs de-esterify pectin, retain high molecular weight, create greater negative ζ-potential, and create different charge distributions. Based on elution of IEX, chemical shift in NMR, and ζ-potential, we observed a block-wise de-esterification pattern following a 10% decrease in DE. Namely, elution from IEX, the peak of B-Pec and U-Pec widened and shifted to a higher ionic strength, indicating an increased charge density. In addition, the negative ζ-potential of B-Pec and U-Pec was greater than O-Pec at the same pH value. Negative ζ-potential was affected by blockwise charge distribution from PME deesterification. Finally, we confirmed that the different modified action pattern between B-PME and U-PME. Also a blockwise structure was deduced based on the 2-fold increase in the F_{GGG} fraction which is an indicator of a block structure. U-Pec has less contiguous blocks of de-esterified pectin than B-Pec, but a greater part of the pectin population was affected. We concluded that based on results from NMR, IEX, and ζ – potential, B-PME and U-PME has multi-attack and multi-chain pattern for modifying pectin. However, U-

PME produces shorter attacks and affected more pectin chains than B-PME. Moreover, the calcium sensitivity of Valencia PME modified pectins led to a better insight into the effect of different pattern of ester distribution on functional properties of B-Pec and U-Pec. First, in the presence of 35 mM $CaCl_2$, 2% B-Pec and U-Pec formed a gel even at high %DE. In contrast, O-Pec did not gel. In addition, the ζ-potential, TPA, and rheology studies confirmed the difference between B-Pec and U-Pec as well as fractions from IEX. Namely, based on results from ζ-potential, TPA, and rheology study, we concluded that B-Pec and U-Pec, with similar ester content, have different gelling properties because of unique pattern of de-esterification that provide a unique charge distribution and population of pectin that is de-esterified. U-Pec which has shorter de-esterified block over more pectin chains showed more effective calcium sensitivity than B-Pec with longer de-esterified blocks affecting fewer pectin chains. Finally, interaction of individual casein fractions with charge modified pectins in dispersed systems gives an idea of the development of tailored pectins for stabilization of milk proteins in specific systems. Each casein fraction interacted uniquely depending on modified pectins. Based on particle size, ζ-potential, and viscosity, U-Pec had the greatest effect on dispersions than B-Pec or O-Pec. κ-casein is distinguished from other casein fractions.

Ultimately, the availability of different enzymes could enhance the structural characterization of pectins and correlation with functional properties. The information would be potentially useful in developing novel pectins for applications such as a stabilizer in acidified dairy products.