STRUCTURE-FUNCTION CHARACTERIZATION OF FRACTIONATED SUGAR BEET PECTIN

by

DEEPIKA ARUN KARNIK

(Under the Direction of Louise Wicker)

ABSTRACT

Sugar beet pectin (SBP) has good emulsifying capacity but does not provide good stability as compared to gum arabic. It was thus fractionated using successive isopropanol increments to produce fractions (F1-F6), unique with respect to protein, ferulic acid, uronic acid, relative fluorescence intensity, particle size, ζ-potential and % degree of esterification. Unfractionated SBP was used as control. F1 and F4 were high (209±13 µg/mg alcohol insoluble solids (AIS); 93±20 µg/mg AIS) and low (77±9 µg/mg AIS; 14±6 µg/mg AIS) respectively in protein and ferulic acid. The surface hydrophobicity (S_o) was nearly 1.3 to 230 times higher in F1 than control or F4, respectively. Thus lower amount of isopropanol produced fractions with greater S_o. Medium chain triglyceride (MCT) emulsions showed lower D[4,3] values with F4 (0.67-0.93 µm) as compared to F1 (1.28-1.42 µm) and thus there was no simple relationship between protein content and emulsifying properties. The emulsions were also analyzed for rheological properties.

INDEX WORDS: Sugar beet pectin, Isopropanol, Fractionation, Protein, Ferulic acid, Uronic acid, Particle size, Zeta Potential, Surface hydrophobicity, Emulsions, Medium chain triglyceride.

STRUCTURE-FUNCTION CHARACTERIZATION OF FRACTIONATED SUGAR BEET PECTIN

by

DEEPIKA ARUN KARNIK

B.Sc., University of Mumbai, India, 2008

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

MASTER OF SCIENCE

ATHENS, GEORGIA

2012

STRUCTURE-FUNCTION CHARACTERIZATION OF FRACTIONATED SUGAR BEET PECTIN

by

DEEPIKA ARUN KARNIK

Major Professor:	Louise Wicker
Committee:	Rakesh Singh
	Casimir C.Akoh

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2012

DEDICATION

I would like to dedicate this thesis to my parents (Sandhya and Arun) and sister (Dr.Tejashree Karnik) for always believing in me and encouraging me. And to my aunt and uncle (Nutan and Prashant) for being so supportive.

ACKNOWLEDGEMENTS

I would like to thank Dr. Louise Wicker for her support, encouragement and guidance throughout my time here at UGA. I would also like to thank my committee members Dr. Casimir Akoh and Dr. Rakesh Singh who were always willing to help and give valuable inputs. Also, thank you to my wonderful lab mates and colleagues who were great help with any problems I faced in the project: Dr. Christina Castlejohn, Dr. Samantha Hawkins, Hannah Akinosho, Dr. Phodchanee Tina, Dr. Jiyoung Jung, Priyadarshi Puranjay, Haiqin Dong and Dr. John Shields.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Pectin from sugar beet is different from citrus and apple pectin because of the emulsification properties it shows rather than conventional gelling and thickening properties. The reason why it shows properties different than the rest is its unique structure with higher amounts of hydrophobic acetyl groups (Michel et al. 1985) on the galacturonic acid chain, more number of hairy regions, more phenolic esters of ferulic acid and higher amount of protein covalently linked (Kirby, MacDougall and Morris 2008) to the hairy regions. The hydrophobic groups reduce interfacial tension and coat oil droplets while the carbohydrate chain increases viscosity and stabilizes by steric repulsions. Although sugar beet pectin (SBP) has good emulsifying capacity, it shows poor emulsion stability in oil-in-water emulsions. So, while SBP would be very useful to make emulsion based products like salad dressings, with health promoting oils, it would be of no use if it is unable to yield a product that is stable for months. Due to this problem, many studies have attempted to characterize the structure of SBP (Williams et al. 2005; Buchweitz 2011; Funami et al. 2008; Garna et al. 2011). The studies indicate that protein moiety plays an essential role, but results are not definitive. Since the functional property is undoubtedly linked to SBP structure, identification of the structure or structures responsible for function could yield fractions with different functionalities for tailored applications.

In the first study of this thesis, SBP was fractionated using successive increments of isopropanol. Six precipitates (fractions F1-F6) were obtained. Each fraction was characterized for protein, ferulic acid, uronic acid, relative fluorescence intensity, particle size, ζ-potential and

degree of esterification to confirm that they were structurally unique. The unfractionated SBP was used as a control and showed different structural characteristics than the fractions. At lower concentrations of alcohol, more hydrophobic fractions were separated. F1 was rich in protein and ferulic acid and F4 was poor in protein and ferulic acid.

In the second study, fractions F1, F4 and the control were used to study the emulsification properties in oil-in-water emulsions. Medium chain triglyceride (MCT) oil was used as the oil phase. This oil is a potential neutraceutical for products like salad dressings. The emulsions were tested for emulsification capacity on day 0 and emulsion stability over 10 days at an increased temperature.

References

Buchweitz, M. (2011). Characterization of sugar beet pectin fractions providing enhanced stability of anthocyanin- based natural blue food colorants. *Food Chemistry, 132,* 1971-1979.

Funami, T., Nakauma, M., Noda, S., Ishihara, S., Al-Assaf, S., & Phillips, G. O. (2008). Enhancement of the performance of sugar beet pectin as an emulsifier. *Foods Food Ingredients Journal, 213,* 347-356.

Garna, H., Emaga, T. H., Robert, C. & Paquot, M. (2011). New method for the purification of electrically charged polysaccharides. *Food Hydrocolloids, 25,* 1219-1226.

Kirby, A. R., MacDougall, A. J., & Morris, V. J. (2008). Atomic force microscopy of tomato and sugar beet pectin molecules. *Carbohydrate Polymers, 71,* 640-647.

Michel, F., Thibault, J.-F., Mercier, C., Heitz, F., & Pouillaude, F. (1985). Extraction and characterization of pectins from sugar beet pulp. *Journal of Food Science, 50,* 1499-1502.

Williams, P. A., Sayers, C., Viebke, C., & Senan, C. (2005). Elucidation of the emulsification properties of sugar beet pectin. *Journal of Agricultural and Food Chemistry, 53,* 3592-3597.

CHAPTER 2

LITERATURE REVIEW

Introduction

Pectins are complex hetero-polysaccharides which are mainly used as gelling and thickening agents in the food industry. They may also be used for cloud stabilization in acidified milk beverages. Pectins have a α-1, 4-galacturonic acid backbone, with α-1, 2 linked L-rhamnose residues inserted within it (smooth regions). Side chains of L-arabinose and D-galactose (hairy regions) arise from the C-4 and C-3 positions of the L-rhamnose residues. The galacturonic acid backbone is partially methyl esterified. When the galacturonic acid backbone has a degree of esterification more than 50%, it is called a high methoxy pectin. At acidic pH and high concentration of sugar, the repulsions between the polymer chains of high methoxy pectins decrease (Morris et al. 1980) and gel is formed. Low methoxy pectins have degree of esterification of the galacturonic acid backbone less than 50% and form gels in the presence of calcium, due to a well understood mechanism. The food industry uses pectin from apple pomace, citrus peels and sugar beet as the major sources.

Structure of sugar beet pectin

Sugar Beet Pectin (SBP) is very different from the apple and citrus pectin because of the presence of higher amounts of hydrophobic acetyl groups (Michel et al. 1985) on the C-2 and C-3 positions of the galacturonic acid chain (Keenan et al. 1985) , more number of hairy regions , more phenolic esters of ferulic acid (Colquhoun et al. 1994, Ralet et al. 1994) and higher amount

of protein covalently linked (Kirby et al. 2008) to the hairy regions. It is also characterized by very low molecular weight (Michel et al. 1985).

Kirby et al. (2008) used an advanced technique of Atomic Force Microscopy (AFM) to create images of the structure of SBP molecules. They found tadpole-like structures which had rigid, unbranched pectin chains linked on one side to the globular protein. They also found that alkali treatment could separate the protein from the pectin chain but did not degrade the protein.

Sugar beet pectin as an emulsifying agent

SBP has emulsifying properties. The proposed mechanism by which SBP acts as an emulsifying agent is that the hydrophobic moieties like protein adsorb and anchor on to the oil droplets and reduce the interfacial tension between the oil and water interface (Funami et al. 2007). The carbohydrate moiety stabilizes by steric and viscosity effects in the aqueous phase. SBP has been looked upon as an alternative to gum arabic, a very efficient food microencapsulating agent (Drusch 2007). The reason is that gum arabic needs to be used in larger quantities (~15%). SBP can be used in smaller quantities (~1.5 - 3%). Also, SBP has an extended configuration and a semi-flexible chain with radius of gyration of 44nm (Funami et al. 2007) whereas gum arabic has a globular, coil configuration with radius of gyration of 28nm(Castellani et al. 2010). So, SBP can cover the oil droplets better than gum arabic, at the lower concentration. In other words, 2% SBP can produce the same reduction in interfacial tension as 15% gum arabic (Leroux et al. 2003, Akhtar et al. 2002).

Leroux et al. (2003) have shown that SBP can produce emulsions with smaller droplet sizes, narrow particle size distributions and more stable emulsions than citrus pectin at the same concentrations of both pectins. On studying the adsorbed fractions of the respective pectins, they

also found that protein and acetyl content was especially high in the adsorbed fractions. Thus, they concluded that higher protein content in SBP was a major reason why it was required in such smaller amounts (2%) as compared to citrus pectin (4%).

The fraction of SBP adsorbed on to the oil droplets is rich in protein and ferulic acid (Williams and Siew 2008). Also, the ratio of galacturonic acid to side chains has been found to be low in the adsorbed fraction (Williams and Siew 2008). It is proposed that the positively charged protein moieties interact with the negatively charged galacturonic acid and form multi-layers at the surface of the oil droplets (Williams and Siew 2008, Siew et al. 2008). Many studies have shown protein as an important factor causing emulsification but there is no simple relationship established (Siew et al. 2008, Williams et al. 2005). It could be different combinations of moieties producing different emulsification results (Siew et al. 2008). Emulsification properties are influenced by the accessibility of protein and ferulic acid to the surface of the oil droplets, proportion of ester groups and molecular mass distribution of the fractions (Williams et al. 2005). Larger molecular weight (MW) fractions ranging from 306 kDa to 562 kDa, gave larger droplet sizes and unstable emulsions (Williams et al. 2005).

Other than an emulsifying agent, SBP has been used to make protein-polysaccharide biopolymer particles (McClements 2010) with β-lactoglobulin (McClements 2009). The protein-SBP complexes are formed by electrostatic interactions and were pH and temperature dependent. These biopolymer particles can mimic lipid droplets and can be used as fat replacers.

Protein in sugar beet pectin

Different studies have shown different amounts of protein contents in SBP. These differences in composition are obvious because of natural diversity in the polysaccharide and

differences in the extraction methods (Yapo et al. 2007a) used and also the method used to estimate protein content. But, the reported values are consistently within a higher range as compared to the protein content in citrus and apple pectin. Some reported values are, 8.6% (Kirby et al. 2008); 5.38% (Funami et al. 2011); 10.4% (Thibault 1988); 2% (Leroux et al. 2003); 3.7% (Williams et al. 2005); and 5.2% (Mesbahi, Jamalian and Farahnaky 2005).

The type of linkage between protein and SBP is still not very clear and many suggestions have been made in the past. Studies have indicated the presence of either a stable complex or a covalent bond between the protein moiety and pectin. It is very difficult to separate the protein moiety from the carbohydrate chains and thus it has been suggested that a stable complex between protein and pectin is present (Kirby et al. 2008, Gromer et al. 2010). On the other hand, covalently linked arabinogalactan-proteins have been suggested to be present in pectin extracted from hops (Oosterveld, Voragen and Schols 2002). The study on cotton pectin by (Qi et al. 1995) also points toward a covalent linkage between the extensin protein and pectin. (Kirby et al. 2008) observed using AFM that ion exchange chromatography could not remove the protein from the pectin. Therefore, the possibility of an ionic complex was ruled out. Also (Nunez et al. 2009) observed that 1M NaCl was not able to separate the protein from the SBP and thus concluded that the linkage was definitely not of ionic nature. (Nunez et al. 2009) used proteomics to identify the protein associated with SBP as extensin. The amino acid profile of the protein has shown hydroxyproline as the most abundant amino acid present. (Funami et al. 2007) made emulsions with 15% MCT oil and 1.5% SBP both unmodified and modified with proteinases. The modified SBP with reduced protein content (1.56±0.15% to 0.13±0.02%), molecular weight (517±28 to 254±20 kg/mol) and radius of gyration (43.6±0.8 to 35.0±0.6 nm) gave emulsion with increased droplet size (0.56±0.04 to 3.00±0.25nm). It also caused creaming and had much

less amount than usual of pectin fraction (14.58±2.21% to 1.22±0.03%), adsorbed on the oil phase. These results indicated that the protein moiety in SBP contributed significantly to the emulsifying property.

Funami et al. (2011) characterized SBP by structural modification using enzymes like protease to degrade the proteinaceous moiety, polygalacturonase to cleave the carbohydrate backbone, and the mixture of arabinanase/galactanase to cleave the lateral chains. This "enhancement" was done in order to assess the contribution of each of these structural units to emulsification and obtain information about structure-function correlation of SBP as an emulsifier. They observed that the loss of protein caused maximum loss of emulsification capacity, followed by the side chains and the backbone cleavage. Cleaving the side chains reduced both the protein content and the ferulic acid content. Also, the protease and arabinase/galactase cleaved SBP produced emulsions with larger droplet sizes and lower creaming stability. This study throws light on the importance of individual components of SBP to emulsion activity and stresses that protein moiety surely dominates this function.

Leroux et al. (2003) studied SBP emulsions made with orange oil and rapeseed oil. They analyzed the SBP remaining in the aqueous phase and the oil phase. The oil phase had a lot more protein than the aqueous phase and concluded that protein played an important role in emulsification.

In spite of so many studies pointing to the importance of protein content, an interesting point to note is the one discussed by (Funami et al. 2007). They reported that it was not enough to conclude that more protein content would provide better emulsification properties. It is not possible to predict the emulsification property of a SBP fraction based on its protein content. The reason suggested was that the molecular arrangement of the protein moiety in relation to the

carbohydrate moiety together plays an important role. The two moieties together influence the hydrophilic-hydrophobic balance of the SBP molecule and may function differently than they would have alone. The adsorption of protein moiety on the oil droplet can be retarded by the carbohydrate portion of a hydrocolloid (Castellani et al. 2010). Therefore, hydrocolloids are required in more concentration as compared to pure proteins for use as an emulsifier (Castellani et al. 2010). Moreover protein folding may hide some hydrophobic functional groups within the protein, thus masking the hydrophobic effect the protein is expected to show (Nakai 1983). Also, for SBP in particular, the weight-average molar mass matters more than the protein content for good emulsification properties. Lower weight-average molar mass is more favorable for emulsification properties.

Ferulic acid in sugar beet pectin

Ferulic acid is another hydrophobic portion present in the structure of SBP. In general, Ferulic acid is found esterified to plant cell wall polysaccharides (Colquhoun et al. 1994). NMR studies have shown ferulic acid linked to C-2 of the L-arabinose residues and C-6 of the D-galactose residues in SBP (Colquhoun et al. 1994). About 30% of the feruloyl groups are carried by the arabinose side chains (Guillon and Thibault 1990). The arabinose side chains are (1→5) linked and may have other arabinose residues or chains linked to C-2 or C-3 positions whereas the galactose chains are (1→4) linked (Colquhoun et al. 1994).

Guillon and Thibault (1990) tried to induce gelation ability in SBP using persulfate and cross-linking of feruloyl groups. Since the feruloyl groups are linked to the main core of the arabinose neutral sugar side chains, they are not easily accessible for cross-linking. The authors could improve gelling ability by removing these peripheral unferuloylated arabinose residues.

Acetyl groups in sugar beet pectin

Methyl ester groups and acetyl groups also provide hydrophobic character to pectin in general (Burapapadh et al. 2010, Dea and Madden 1986). These groups make SBP amphiphilic and provide the ability to adsorb or spread on oil droplets (Burapapadh et al. 2010).

Leroux et al. (2003) found that acetylated citrus pectin gave better emulsifying ability than non-acetylated. Acetyl groups can lower the calcium sensitivity and thus contribute to emulsion property (Leroux et al. 2003). They proposed that acetyl groups must be contributing to emulsion stability by reducing calcium bridging flocculation. But, when they deacetylated SBP, there was no significant loss of emulsification capacity.

Also, acetyl groups prevent gel formation by SBP (Michel et al. 1985). De-acetylation allows gel formation property in SBP (Dea and Madden 1986). Matthew et al. (1990) proposed that removal of a small number of acetyl groups that normally prevent calcium bridge formation by steric effects, may improve the gelling ability of SBP. They deacetylated SBP using enzymatic and chemical methods and found that slight deacetylation improved the gelling ability. Matora et al. (1995) have also shown that high acetyl content can lead to loss of gel forming ability in pumpkin and SBPs. They extracted the pectin using bacterial enzymes instead of the conventional acid extraction. The method gave high yields of pectin which had acetyl content higher than usual. These products had no gelling properties at all.

Use of ANS for surface hydrophobicity

ANS is 8-Anilino-1-naphthalene-sulfonic acid. It is a hydrophobic fluorescent dye and is negatively charged. It has increased fluorescence in less polar environments (Fukunaga et al. 2008). Proteins are hydrophobic in nature and can provide hydrophobic binding sites to ANS.

The amount of fluorescence that the dye can produce depends upon the accessible hydrophobic chains, viscosity and also the electrostatic interactions between the molecules (Qu et al. 2009).

Use of Fourier Transform Infrared Spectroscopy with pectin

Fourier Transform Infrared Spectroscopy (FTIR) is a very quick way to study the structure of pectins (Chatjigakis et al. 1998). Specific absorptions can be assigned to different functional groups and thus the FTIR can be used to characterize the structure of different materials (Kacurakova and Wilson 2001). The absorptions are usually measured in the frequency range of 4000- 400cm^{-1}(Kacurakova and Wilson 2001). Intensity of absorption can give an idea about the quantitative nature of different functional groups (Kacurakova and Wilson 2001, Kamnev et al. 1998). Because of the complexity of pectins, it is not possible to assign very specific band numbers for each group in the IR spectra. But differences in the presence, absence and intensity of certain range of bands can be used to characterize them (Kacurakova and Wilson 2001). The complexities may arise due to differences in polymer conformation or individual macromolecular interactions (Kacurakova and Wilson 2001).

Previous attempts to fractionate or modify sugar beet pectin

SBP has shown poor emulsion stability as compared to other food hydrocolloids. SBP has been subjected to fractionation in a previous study by (Williams et al. 2005). They used hydrophobic affinity chromatography for fractionation. All the fractions differed in protein, ferulic acid and molecular weight. The low molecular weight (153 kDa-282 kDa) fractions were able to make emulsions with smaller droplet sizes and were stable for a longer time as compared to the high molecular weight (306 kDa-562 kDa) fractions. The relationship between molecular

weight and droplet size of emulsions has been verified by (Yapo, Wathelet and Paquot 2007b) also. They used 1% SBP and 30% oil. The D[4,3] values for the emulsions prepared with the 35, 46, 70, 82, 139, and 141 kg/mol pectin fractions were 1.08, 0.82, 0.92, 1.01, 3.25, and 3.31 µm, respectively, after 24 h of aging at room temperature. Also (Williams et al. 2005) could not draw a firm conclusion about the relationship between protein and ferulic acid content and emulsification property. This uncertainty has been verified by (Funami et al. 2007). It was clear enough that protein was adsorbed on to the oil-water interface and its role was influenced not only by the actual content but also its structural relationship with other components.

Buchweitz (2011) successively used 80, 70, 70 and 70% aqueous isopropanol to precipitate SBP. At each step, a precipitated fraction was obtained. The fractions were analyzed for neutral sugars using gas chromatography; uronic acid using m-hydroxydiphenyl method; methyl, acetyl and ferulic acid contents using HPLC; and molecular size distribution using high-performance size exclusion chromatography. Fractions 3 and 4 were blended in equal amounts to produce a pectic polysaccharide fraction (PPF). The PFF was further tested for stability of blue color of anthocyanins at lower pH values. It was found to provide stability to blue color for the same. This study shows that isopropanol was successively used to wash and precipitate SBP, yielding fractions with unique structures and validates the idea of using alcohol to fractionate pectin, as done in chapter 3 of this thesis.

Another attempt to modify SBP has been done by (Funami et al. 2008). They used a maturation process which caused the proteins to aggregate and act as linkers to associate the carbohydrate chains. This formed a bulky carbohydrate layer around the oil droplets. The bulky carbohydrate provided good steric stabilization and it was difficult to disaggregate the proteins. This prevented flocculation for a long time and improved emulsion stability. The process also

produced orange oil and lime oil emulsions that were stable to thermal sterilization, unlike the untreated control SBP.

Garna et al. (2011) came up with a new method of extracting SBP from sugar beet pulp using sodium caseinate protein, instead of ethanol extraction. In this method, it was possible to get pectin products of desired compositions by controlling ionic strength. At pH 3.5, there were strong electrostatic interactions between sodium caseinate and pectin and thus pectin was precipitated. The protein was then separated from SBP by raising the pH to 4.6.

Medium chain triglyceride (MCT) oil

MCT have a fatty acid chain length between 6 to 10 carbon atoms. Unlike long chain triglycerides, they are relatively soluble in water and are converted to medium chain fatty acids (MCFA) and transported to the portal venous system directly. They are not transported to the lymphatic system. They thus do not reach the peripheral adipose tissue and are not deposited there. This can help control weight as opposed to consuming long chain triglycerides. They have also been shown to improve satiety possibly because of quicker breakdown of MCT into MCFA; increased production of ketones which suppress hunger; and all MCT getting broken down faster in one meal unlike LCT that break down over the first and the next meal. Also, because of the small amount of time they spend in the gastrointestinal tract, they can be used as enteral and parenteral nutrition for people with fat malabsorption problems. The main sources of MCT are coconut and palm (Clegg 2010).

Some studies have suggested that MCT can provide readily available fat for oxidation during exercise and can spare glycogen in the body which is required for greater stamina in athletes (Visiten et al. 2003, Nosaka et al. 2009) but many other studies have not been able to

prove any positive effect of MCT on exercise performance (Misell et al. 2001, Goedecke et al. 2005). Some studies have also combined MCT with a carbohydrate to see the effect on exercise performance and it has not proved to be promising for the same (Jeukendrup et al. 1998). Thus research has been aimed more at the health benefits of MCT than the performance exercise it may provide and MCT is a potential neutraceutical that the food industry may use in many food emulsions.

Emulsions

Dickinson (2003) defined an emulsifying agent as a chemical species that can cause emulsion formation and can stabilize it for a short amount of time by interfacial action. They are amphiphilic molecules. Emulsifying agents can be small molecules or macromolecules (Dickinson 2003). On the other hand, stabilizing agents provide long term stability by viscosity or gelling changes. An important property of emulsifying agents is to be surface-active (Dickinson 2003). A surface active species works by lowering the surface tension at the oil-water interface in an oil-in-water emulsion (Dickinson 2003).

During emulsion formation, the main step is high pressure homogenization and it consists of droplet formation, adsorption of emulsifier on the droplet, spreading of the emulsifier on the droplet and collision of droplets (Dickinson 2003). An emulsifier with good surface-active property can spread faster than the time taken for droplet collisions (Dickinson 2003). An emulsifier is expected to have small molecular size and good solubility in water, in order to give small droplet size in the emulsion. Dickinson (2003) explained that large molecular size emulsifiers may require a long time to spread on the oil droplets as compared to the time taken for droplet collision during high pressure homogenization. In other words, large molecular size

of emulsifier delays the reduction of surface tension at the oil-water interface. Leroux et al. (2003) reported that too low molecular weight pectin gave coarse emulsions with undesired droplet sizes and stability. Also, too high molecular weights caused calcium bridging flocculation and did not give the best droplet sizes. A range of 50-80 kg/mol was identified as a good size, giving small droplet sizes and good stability.

Another desirable property of the emulsifier should be a charged hydrophilic component which should be able to provide stabilizing action by charge repulsion (Dickinson 2003). It is also important to use the emulsifier at an optimum concentration.

Destabilization mechanisms in emulsions

The common mechanisms by which oil-in-water emulsions can destabilize are gravitational separation, flocculation, coalescence, Ostwald ripening and phase inversion (McClements 2007). Gravitational separation is the process by which droplets move upward causing creaming because they have a lower density than the surrounding liquid, or downwards causing sedimentation because they have a higher density than the surrounding liquid. Flocculation is the process whereby two or more droplets stick together to form aggregates in which each of the initial droplets retains its individual integrity. When oil-in-water emulsions have added polysaccharides, they can induce depletion flocculation or bridging flocculation (Blijdenstein et al. 2004). Depletion flocculation occurs when there is excess of polysaccharide in the emulsion (Blijdenstein et al. 2004). The polysaccharides fail to bind to the oil droplets and start to deplete from the droplet surfaces. This depletion of polysaccharide from the oil droplets' surface gives rise to attraction between them and causes flocculation. Bridging flocculation on the other hand takes place when the amount of polysaccharide is less then optimum. A single

molecule of polysaccharide adsorbs on to two or more oil droplets. A polysaccharide bridge is formed between the participating oil droplets and an irreversible electrostatic bond is formed between the oil droplets, leading to flocculation. Depletion flocculation forms weak and reversible bonds (Blijdenstein et al. 2004). Coalescence is the process whereby two or more droplets merge together to form a single droplet larger than the merging droplets. Ostwald ripening is the process by which larger droplets grow at the expense of smaller droplets due to mass transport of dispersed phase material through the continuous phase. Phase inversion is the process whereby an oil-in-water emulsion changes to a water-in-oil emulsion, or vice versa.

Behavior of sugar beet pectin in emulsions

The % oil content and % SBP content have been shown to influence the median droplet size of an emulsion (Drusch 2007). Increasing pectin concentration up to a critical value decreases the droplet size of emulsions (Burapapadh et al. 2010). The critical concentration of pectin does influence the stability of an emulsion (Burapapadh et al. 2010). Also, pectin increases the viscosity of the solutions and slows down the droplet movement in emulsions (Parker et al. 1995). Acidic pH creates aggregations at the air-water interface of the hydrocolloid films. These aggregations improve the spreading capacity of the hydrocolloid on the oil droplets (Castellani et al. 2010).

Gromer et al. (2009) studied the structure of SBP in emulsions, at different interfaces using AFM. They studied adsorption of SBP on mica and graphite which are hydrophilic and hydrophobic respectively. From this they could characterize the way in which protein and pectin in SBP would align themselves on a hydrophobic and a hydrophilic surface. They also studied the SBP film at the air-water interface using Langmuir-Blodgett method and concluded that the

proteins in the SBP cannot pack tightly because of the presence of pectin chains. From surface shear rheology, they concluded that SBP films are elastic in nature. Also, surface tension of the SBP films was measured after alkali treatment and it was concluded that alkali treatment separated the protein moiety from pectin and formed fibers. The result was a fibrous film, containing only protein. Thus SBP film showed more resistance to alkali treatment than a pure protein solution would show. This detailed information is a potential step towards more studies on modifying SBP structure and hoping to get new or improved functional properties.

Gromer et al. (2010) used AFM to study the behavior of SBP with oil droplets in actual emulsions, at a molecular level. They used pendant drop method to measure the interfacial tension between the tetradecane/water interface. The interfacial tension fell for many hours and did not increase. So, the binding of SBP was irreversible. Also, the elastic modulus (G') measured by shear rheology was greater than G" and had values similar to that of protein films. Thus, it proved that SBP formed elastic films at the oil/water interface. The interfacial films were transferred on mica and studied using AFM. 0.1% SBP formed a 0.9nm thick layer with holes. It was concluded that the elastic nature of the SBP created holes by preventing lateral diffusion. At 0.5% SBP concentration, the holes were filled up and there was closer packing of pectin chains. At 2% SBP concentration, the interface was saturated with SBP, appeared rough and did not allow individual pectin chains to be identified.

Markman and Livney (2012) found that the conjugating casein protein with oligosaccharides made the hydrophilic portions of the protein bulky and decreased their packing parameter. This increased the droplet curvature of the encapsulated hydrophobic bioactive compound and gave smaller size of encapsulated droplets. Also, the saccharide layer formed a

protective outer shell for stabilization. This mechanism may be playing a role in better emulsifying properties associated with low protein-high carbohydrate SBP fractions.

Methods used to study emulsions

An emulsion is said to be stable when the droplet size distribution remains the same during the time it is stored and when the droplets do not show any aggregation due to coalescence, flocculation, Ostwald ripening or gravity creaming (Dickinson 2003). The morphology of emulsions has been studied by placing an emulsion drop on a glass slide, covering it with a coverslip and taking pictures under a biological light microscope (Burapapadh et al. 2010). Microscopy has also been used to measure diameter of the emulsion droplets (Burapapadh et al. 2010). Another method for calculating emulsion droplet size is by laser diffraction method, where the droplet size is measured by diluting the emulsion with de-ionized water and is continuously stirred (Burapapadh et al. 2010). Since the droplet sizes of SBP emulsions are micron sized, the mastersizer is suitable to measure them. A narrow distribution width indicates a good emulsion with uniformly sized droplets. Increase in the distribution width indicates unfavorable changes in the emulsions. Relative span is one of the ways to represent distribution width. Relative span is given by $(d_{90} - d_{10})/d_{50,}$ where d_{90} is the diameter below which 90% of the droplets can be placed, d_{10} the diameter below which 10% of the droplets can be placed, and d_{50} the diameter below which 50% of the droplets can be placed. The smaller the droplet diameters on day 0, the better the emulsification capacity. The presence of an additional peak in the droplet size distribution curve, rather than a perfect Gaussian curve is due to the presence of calcium, which causes bridging flocculation (Leroux et al. 2003). Addition of a strong surfactant to a stored pectin- stabilized emulsion removed the additional peak and also

showed reduction in the D[4,3] value. This proved that the surfactant was able to take over the flocculated droplets and that pectin was quite resistant to coalescence. Flocculation was a major reason for instability in pectin stabilized emulsions (Akhtar et al. 2002). The conclusions from droplet size distribution can be verified using microscopy images.

Rheology is a very good method to study the flow behavior of emulsions. According to (Meriem-Benziane et al. 2012), stable viscosity with increasing shear rates represents a quasi-Newtonian behavior and changing viscosity with increasing shear rate represents non-Newtonian behavior. Viscosity changes of emulsions with changing shear rates have been studied. When viscosity decreases with increasing shear rate, the emulsion is said to have non-Newtonian behavior and is said to be shear-thinning. When the viscosity increases with increasing shear rate, the behavior is called shear-thickening. It is more common to observe shear-thinning among non-Newtonian fluids. The decrease in viscosity may be due to breaking up of aggregated particles at higher shear rates (Kim 2002). But when the viscosity remains constant with change in shear rate, the behavior is said to be quasi-Newtonian (Meriem-Benziane et al. 2012). The range of shear rate applied to foods is usually $10^1 – 10^2\ s^{-1}$ (Barnes, Hutton and Walters 1989).

Some rheological laws and models have been used to characterize the viscosity behavior of emulsions (Meriem-Benziane et al. 2012, Ercelebi and Ibanoglu 2009). The rheological laws like Power Law, Herschel-Bulkley model and Casson model can give useful information about the colloidal suspension of emulsions (Hasan, Ghannam and Esmail 2010). The power law model is given by $\eta=m\gamma^{n-1}$, where m is consistency and directly proportional to viscosity, η is the apparent viscosity and n measures the non-Newtonian behavior. n=1 indicates Newtonian behavior, n<1 indicates shear thinning and n>1 indicates shear thickening. Power law can be generally applied to all non-Newtonian fluids. But the behavior of non-Newtonian fluids with

yield stress (viscoplastic fluids), for example emulsions, can be fit into models like Bingham plastic model, Herschel-Bulkley model and Casson model. Bingham plastic model cannot handle shear thinning or shear thickening behavior because it can be applied only when viscosity is independent of shear rate. Casson model indicates yield stress and shear thinning or thickening behavior of non-Newtonian fluids. The Herschel-Bulkley model is a modification of the power law which includes yield stress. The Casson model and Herschel-Bulkley model assume the emulsions to be rigid three-dimensional structures. Above the yield stress, the viscosity changes and the emulsions start flowing at higher shear rates. Yield stress is the stress below which the material behaves like a solid and above which the material deforms and flows. Emulsions like salad dressings are good examples of materials having yield stress. R^2 has been used as a tool to validate the models for the emulsions. The closer R^2 is to 1, the better fitting the model.

SBP has potential to show improved emulsification and stabilization properties. Some of the many attempts to modify SBP have been successful and there is room for lot more research on improving its stabilization property. There are many methods used to characterize the structure of this hydrocolloid and also to study its behavior in emulsions. The results of modification and fractionation of SBP can thus be easily studied. This study is aimed at finding out if there can be a definite relationship between hydrophobic groups and emulsification activity of new fractions obtained by isopropanol fractionation and if the hydrophobic fractions can be used to make stable oil-in-water emulsions having neutraceutical value.

References

Akhtar, M., Dickinson, E., Mazoyer, J., & Langendorff, V. (2002). Emulsion stabilizing properties of depolmerized pectin. *Food Hydrocolloids, 16,* 249-256.

Asboe-Hansen, G. & Blumenkrantz, N. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry, 54,* 484-489.

Barnes, H. A., Hutton, J. F., & Walters, K. (1989). *An introduction to rheology.* Amsterdam: Elsevier.

Blijdenstein, T. B. J., Winden, A. J. M., Vliet, T. v., v., Linden, E. v. d., & Aken, G. A. v. d. (2004). Serum separation and structure of depletion- and bridging-flocculated emulsions: a comparison. *Colloids and surfaces A: Physicochemical and Engineering Aspects, 245,* 41-48.

Buchweitz, M. (2011). Characterization of sugar beet pectin fractions providing enhanced stability of anthocyanin- based natural blue food colorants. *Food Chemistry, 132,* 1971-1979.

Burapapadh, K., Kumpugdee-Vollrath, M., Chantasart, D., & Sriamornsak, P. (2010). Fabrication of pectin-based nanoemulsions loaded with itraconazole for pharmaceutical application. *Carbohydrate Polymers, 82,* 384-393.

Castellani, O., Al-Assaf, S., Axelos, M., Phillips, G. O., & Anton, M. (2010). Hydrocolloids with emulsifying capacity. Part 2- adsorption properties at the n-hexadecane-water interface. *Food Hydrocolloids, 24,* 121-130.

Chatjigakis, A. K., Pappas, C., Proxenia, N., Kalantzi, O., Rodis, P., & Polissiou, M. (1998). FT-IR spectroscopic determination of the degree of esterification of cell wall pectins from stored peaches and correlation to textural changes. *Carbohydrate Polymers, 37,* 395-408.

Clegg, M. E. (2010). Medium-chain triglycerides are advantageous in promoting weight loss althoughnot beneficial to exercise performance. *International Journal of Food Sciences and Nutrition, 61,* 653-679.

Colquhoun, I. J., Ralet, M.-C., Thibault, J.-F., Faulds, C. B., & Williamson, G. (1994). Structure identification of feruloylated oligosaccharides from sugar-beet pulp by NMR spectroscopy. *Carbohydrate Research, 263,* 243-256.

Costantino, H. R., Griebenow, K., Langer, R., & Klibanov, A. M. (1997). On the pH memory lyophilized compounds containing protein functional groups. *Biotechnology and Bioengineering, 53,* 348-348.

Dea, I. C. M. & Madden, J. K. (1986). Acetylated pectic polysaccharides of sugar beet. *Food Hydrocolloids, 1,* 71-88.

Dickinson, E. (2003). Hydrocolloids at interfaces and the influence on the properties of dispersed systems. *Food Hydrocolloids, 17,* 25-39.

Drusch, S. (2007). Sugar beet pectin: A novel emulsifying wall component for microencapsulation of lipophilic food ingredients by spray-drying. *Food Hydrocolloids, 21,* 1223-1228.

Ercelebi, E. A. & Ibanoglu, E. (2009). Rheological properties of whey protein isolate stabilized emulsions with pectin and guar gum. *European Food Research & Technology, 229,* 281-286.

Fukunaga, Y., Nishimoto, E., Otosu, T., Murakami, Y., & Yamashita, S. (2008). The unfolding of α-momorcharin proceeds through the compact folded intermediate. *Journal of Biochemistry, 144,* 457-466.

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

Funami, T., Nakauma, M., Noda, S., Ishihara, S., Al-Assaf, S., & Phillips, G. O. (2008). Enhancement of the performance of sugar beet pectin as an emulsifier. *Foods Food Ingredients Journal, 213,* 347-356.

Funami, T., Zhang, G., Hiroe, M., Noda, S., Nakauma, M., Asai, I., Cowman, M. K., Al-Assaf, S., & Phillips, G. O. (2007) Effects of proteinaceous moiety on the emulsifying properties of sugar beet pectin. *Food Hydrocolloids, 21,* 1319-1329.

Garna, H., Emaga, T. H., Robert, C., & Paquot, M. (2011). New method for the purification of electrically charged polysaccharides. *Food Hydrocolloids, 25,* 1219-1226.

Goedecke, J. H., Clark, V. R., Noakes, T. D., & Lambert, E. V. (2005). The effects of medium-chain triacylglycerol and carbohydrate ingestion on ultra-endurance exercise performance. *International Journal of Sport Nutrition and Exercise Metabolism, 15,* 15-27.

Gromer, A., Kirby, A. R., Gunning, A. P., & Morris, V. J. (2009). Interfacial structure of sugar beet pectin studied by atomic force microscopy. *American Chemical Society, 25,* 8012-8018.

Gromer, A., Penfold, R., Gunning, A. P., Kirby, A. R., & Morris, V. J. (2010). Molecular basis for the emulsifying properties of sugar beet pectin studied by atomic force microscopy and force spectroscopy. *Soft Matter, 6,* 3957-3969.

Guillon, F. & Thibault, J.-F. (1990) Oxidative cross-linking of chemically and enzymatically modified sugar-beet pectin. *Carbohydrate Polymers, 12,* 353-374.

Hasan, S. W., Ghannam, M. T., & Esmail, N. (2010). Heavy crude oil viscosity reduction and rheology for pipeline transportation. *Fuel, 89*, 1095-1100.

Jeukendrup, A. E., Thielen, J. J., Wagenmakers, A. J., Brouns, F., & Saris, W. H. (1998). Effect of medium-chain triacylglycerol and carbohydrate ingestion during exercise on substrate utilization and subsequent cycling performance. *American Journal of Clinical Nutrition, 67*, 397-404.

Kacurakova, M. & Wilson, R. H. (2001). Developments in mid-infrared FT-IR spectroscopy of selected carbohydrates. *Carbohydrate Polymers, 44*, 291-303.

Kamnev, A. A., Colina, M., Rodriquez, J., Ptitchkina, N. M., & Ignatov, V. V. (1998). Comparitive spectroscopic characterization of different pectins and their sources. *Food Hydrocolloids, 12,* 263-271.

Karnik, D (2012). Fractionation of sugar beet pectin using isopropanol and characterization of the fractions obtained. Athens: University of Georgia.

Kato, A. & Nakai, S. (1980). Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochimicha et Biophysica Acta., 624,* 13-20.

Keenan, M. H. J., Belton, P. S., Matthew, J. A., & Howson, S. J. (1985). A 13C-n.m.r. study of sugar-beet pectin. *Carbohydrate Research, 138*, 168-170.

Kertesz. (1951). *The pectic substances*. New York: Interscience Publishers.

Kim, S. (2002). A study of non-newtonian viscosity and yield stress of blood in a scanning capillary-tube rheometer. Drexel University.

Kim, Y. & Wicker, L. (2009). Valencia PME isozymes create charge modified pectins with distinct calcium sensitivity and rheological properties. *Food Hydrocolloids, 23*, 957-963.

Kirby, A. R., MacDougall, A. J., & Morris, V. J. (2008). Atomic force microscopy of tomato and sugar beet pectin molecules. *Carbohydrate Polymers, 71,* 640-647.

Leroux, J., Langendorff, V., Schick, G., Vaishnav, V., & Mazoyer, J. (2003). Emulsion stabilizing properties of pectin. *Food Hydrocolloids, 17,* 455-462.

Markman, G. & Livney, Y. D. (2012). Maillard-conjugate based core-shell co-assemblies for nanoencapsulation of hydrophobic nutraceuticals in clear beverages. *Food & Function, 3,* 262.

Matora, A. V., Korshunova, V. E., Shkodina, O. G., Zhemerichkin, D. A., Ptitchkina, N. M., & Morris, E. R. (1995). The application of bacterial enzymes for extraction of pectin from pumpkin and sugar beet. *Food Hydrocolloids, 9,* 43-46.

Matthew, J. A., Howson, S. J., Keenan, M. H. J., & Belton, P. S. (1990). Improvement of the gelation properties of sugarbeet pectin following treatment with an enzyme preparation derived from *Aspergillus niger* — comparison with a chemical modification. *Carbohydrate Polymers, 12,* 295-306.

McClements, D. J. *Food Emulsions: Principles, Practices and Techniques*.

--- (2007) Critical Review of Techniques and Methodologies for Characterization of Emulsion Stability. *Critical Reviews in Food Science and Nutrition, 47,* 611-649.

Meriem-Benziane, M., Abdul-Wahab, S. A., Benaicha, M., & Belhadri, M. (2012). Investigating the rheological properties of light crude oil and the characteristics of its emulsions in order to improve pipeline flow. *Fuel, 95,* 97-107.

Mesbahi, G., Jamalian, J., & Farahnaky, A. (2005). A comparative study on functional properties of beet and citrus pectins in food systems. *Food Hydrocolloids, 19,* 731-738.

Michel, F., Thibault, J.-F., Mercier, C., Heitz, F., & Pouillaude, F. (1985). Extraction and Characterization of Pectins from Sugar Beet Pulp. *Journal of Food Science, 50,* 1499-1502.

Misell, L. M., Lagomarcino, N. D., Schuster, V., & Kern, M. (2001). Chronic medium-chain triacylglycerol consumption and endurance performance in trained runners. *Journal of Sports Medicine and Physical Fitness, 41,* 210-215.

Morris, E. R., Gidley, M. J., Murray, E. J., Powell, D. A., & Rees, D. A. (1980). Characterization of pectin gelation under conditions of low water activity, by circular dichroism, competitive inhibition and mechanical properties. *International Journal of Biological Macromolecules, 2,* 327-330.

Nakai, S. (1983). Structure-function relationships of food proteins with an emphasis on the importance of protein hydrophobicity. *Journal of Agricultural and Food Chemistry, 31,* 676-683.

Nosaka, N., Suzuki, Y., Nagatoishi, A., Kasai, M., Wu, J., & Taguchi, M. (2009). Effect of ingestion of medium-chain triacylglycerols on moderate and high-intensity exercise in recreactional atheletes. *Journal of Nutritional Science and Vitaminology, 55,* 120-125.

Nunez, A., Fishman, M. L., Fortis, L. L., Cooke, P. H., & Hotchkiss, A. T. (2009). Identification of extensin protein associated with sugar beet pectin. *Journal of Agricultural and Food Chemistry, 57,* 10951-10958.

Oosterveld, A., Grabber, J. H., Beldman, G., Ralph, J., & Voragen, A. G. J. (1997). Formation of ferulic acid dehydrodimers through oxidative cross-linking of sugar beet pectin. *Carbohydrate Research, 300,* 179-181.

Oosterveld, A., Voragen, A. G. J., & Schols, H. A. (2002). Characterization of hop pectins shows presence of an arabinogalactan-protein. *Carbohydrate Polymers, 49*, 407-413.

Parker, A., Gunning, P. A., Ng, K., & Robins, M. M. (1995). How does xanthan stabilise salad dressing? *Food Hydrocolloids, 9*, 333-342.

Qi, X., Behrens, B. X., West, P. R., & Mort, A. J. (1995). Solubilization and partial characterization of extensin fragments from cell walls of cotton suspension cultures. *Plant Physiology, 108*, 1691-1701.

Qu, P., Lu, H., Yan, S., Zhou, D., & Lu, Z. (2009). Investigations of effects of environmental factors in unfolding/refolding pathway of proteins on 8-anilino-1-naphthalene-sulfonic acid (ANS) fluorescence. *Journal of Molecular Structure, 936*, 187-193.

Ralet, M.-C., Thibault, J.-F., Faulds, C. B., & Williamson, G. (1994). Isolation and purification of feruloylated oligosaccharides from cell walls of sugar beet pulp. *Carbohydrate Research, 263*, 227-241.

Siew, C. K., Williams, P. A., Cui, S. W., & Wang, Q. (2008). Characterization of the surface-active components of sugar beet pectin and the hydrodynamic thickness of the adsorbed pectin layer. *Journal of Agricultural and Food Chemistry, 56*, 8111-8120.

Socrates. (2001). *Infrared and raman characteristic group frequencies: Tables and Charts*. New York: John Wiley & Sons.

Thibault, J.-F. (1988). Characterization and oxidative crosslinking of sugar-beet pectins extracted from cossettes and pulps under different conditions. *Carbohydrate Polymers, 8*, 209-223.

Visiten, B., Nybo, L., Xu, K., Hoy, C.-E., & Kiens, B. (2003). Minor amounts of plasma medium-chain fatty acids and no improved time trial performance after consuming lipids. *Journal of Applied Physiology, 95*, 2434-2443.

Williams, P. A., Sayers, C., Viebke, C., & Senan, C. (2005). Elucidation of the emulsification properties of sugar beet pectin. *Journal of Agricultural and Food Chemistry, 53,* 3592-3597.

Williams, P. A. & Siew, C. K. (2008). Role of protein and ferulic acid in the emulsification properties of sugar beet pectin. *Journal of Agricultural and Food Chemistry, 56,* 4164-4171.

Yapo, B. M., Robert, C., Etienne, I., Wathelet, B., & Paquot, M. (2007a). Effect of extraction conditions on the yield, purity and surface properties of sugar beet pulp pectin extracts. *Food Chemistry, 100*, 1356-1364.

Yapo, B. M., Wathelet, B., & Paquot, M. (2007b). Comparison of alcohol precipitation and membrane filtration effects on sugar beet pulp pectin chemical features and surface properties. *Food Hydrocolloids, 21*, 245-255.

Zhang, C., Quek, S. Y., Lam, G., & Easteal, A. J. (2008). The rheological behaviour of low fat soy-based salad dressing. *International Journal of Food Science and Technology, 43*, 2204-2212.

CHAPTER 3

FRACTIONATION OF SUGAR BEET PECTIN USING ISOPROPANOL AND CHARACTERIZATION OF THE FRACTIONS OBTAINED[1]

[1] Karnik, D., Hawkins, S. and Wicker, L. To be submitted to Food Hydrocolloids

Abstract

Since isopropanol precipitates pectin, we hypothesized that with increasing lyophilic solvent, protein and ferulic acid rich sugar beet pectin (SBP) fractions would precipitate at lower isopropanol concentrations and produce fractions having higher emulsifying capacity. Isopropanol was sequentially added to 900 mL 2% SBP and precipitate was collected after each addition to obtain six alcohol insoluble solids (AIS). Less than 15% precipitated after the total addition of 200 mL isopropanol and 50% precipitated after total addition of 300 mL isopropanol. Protein was high in Fractions 1 and 2 at 209 and 154 µg/mg AIS, respectively. Fractions 3, 4, and 5 had lower protein values of 77-106 µg/mg AIS. Ferulic acid content in Fraction 1 was 93 µg/mg AIS and at least three times higher than measured in other fractions. The degree of esterification in Fraction 1 was 11% and ranged from 46-74% in later precipitating fractions. The relative surface hydrophobicity measured by an external probe ranged from 159-254 in F1, F2 and F3 compared to negligible values in later fractions. The ζ- potential ranged from -25 to -33 mV and did not change. FTIR analysis confirmed that F1 and F2 were rich in protein. SBP that precipitated at the lowest isopropanol addition had the greatest protein, ferulic acid content, particle size, relative fluorescence intensity and lowest uronic acid content and degree of esterification. Isopropanol fractionation resulted in SBP fractions with unique physico-chemical properties.

INDEX WORDS: Sugar beet pectin, Isopropanol, Fractionation, Protein, FTIR.

Introduction

Pectins are complex hetero-polysaccharides which are mainly used as gelling and thickening agents in the food industry. Generally, pectins have a α-1,4-galacturonic acid backbone, with 1,2 linked α-L-rhamnose residues inserted within it (smooth regions). Side chains of L-arabinose and D-galactose (hairy regions) arise from the C-3 and C-4 positions of the α-L-rhamnose residues. The galacturonic acid backbone is partially methyl esterified.

The food industry uses pectin from apple pomace, citrus peels and sugar beet as the major sources. The reason why sugar beet pectin (SBP) is very different from the apple and citrus pectin is the presence of higher amounts of hydrophobic acetyl groups on the galacturonic acid chain (Michel et al. 1985), more number of hairy regions, more phenolic esters or ferulic acid and higher amount of protein covalently linked to the hairy regions (Funami et al. 2008). It is also characterized by very low molecular weight (Michel et al. 1985). Atomic Force Microscopy (AFM) has been used and SBP molecules have been seen as tadpole-like structures which have rigid, unbranched pectin chains linked on one side to the globular protein (Kirby et al. 2008).

SBP was identified as an equal or superior emulsifying agent compared to gum acacia (Leroux et al. 2003). The proposed mechanism by which SBP acts as an emulsifying agent is that the hydrophobic moieties like protein adsorb and anchor on to the oil droplets and reduce the interfacial tension between the oil and water interface (Funami et al. 2007). The carbohydrate moiety stabilizes by steric and viscosity effects in the aqueous phase.

Different studies have shown different amounts of protein contents in SBP because of natural diversity in the polysaccharide, differences in the extraction methods (Yapo et al. 2007a) used and also the method used to estimate protein content. But, the reported values are consistently within a higher range as compared to the protein content in citrus and apple pectin.

Some reported values are, 8.6% (Kirby et al. 2008); 5.38% (Funami et al. 2011); 10.4% (Thibault 1988); 2% (Leroux et al. 2003); 3.7% (Williams et al. 2005); and 5.2% (Mesbahi et al. 2005).

Emulsions with medium chain triglyceride (MCT) oil and proteinase modified or unmodified 1.5% SBP have been made (Funami et al. 2007). The modified SBP, with reduced protein content, molecular weight and radius of gyration, gave emulsion with increased droplet size. It also caused creaming and the adsorbed phase had much less amount of pectin than usual. These results indicated that the protein moiety in SBP contributed significantly to the emulsifying property.

Non-modified SBP and SBP modified with protease, polygalacturonase and arabinanase/galactanase mixture was used to cleave protein, polygalacturonic backbone and side chains, respectively (Funami et al. 2011). It was observed that the loss of protein caused the maximum loss of emulsification capacity, followed by the side chains and the backbone cleavage. Cleaving the side chains reduced both the protein content and the ferulic acid content. Also, the SBP with protease and arabinase/galactase treatment, produced emulsions with larger droplet sizes and lower creaming stability. This study throws light on the importance of individual components of SBP to emulsion activity and stresses that protein moiety surely dominates this function.

SBP emulsions made with orange oil and rapeseed oil were studied (Leroux et al. 2003) and SBP remaining in the aqueous phase and the oil phase was analyzed. The oil phase had a lot more protein than the aqueous phase and it was concluded that protein played an important role in emulsification.

In spite of so many studies pointing at the importance of protein content, an interesting point to note is that more protein content may not provide better emulsification properties (Funami et al. 2007). It is not possible to predict the emulsification property of a SBP fraction based on its protein content because the molecular arrangement of the protein moiety in relation to the carbohydrate moiety together plays an important role. The two moieties together influence the hydrophilic-lipophilic balance (HLB) of the SBP molecule and may function differently than they would have alone. Also, the adsorption of protein moiety on the oil droplet can be retarded by the carbohydrate portion of a hydrocolloid (Castellani et al. 2010). Therefore, hydrocolloids are required in more concentration compared to pure proteins for use as an emulsifier (Castellani et al. 2010).

The fraction of SBP adsorbed on to the oil droplets is rich in protein and ferulic acid (Williams and Siew 2008). Also, the ratio of galacturonic acid to side chains has been found to be low in the adsorbed fraction (Williams and Siew 2008). It is proposed that the positively charged protein moieties interact with the negatively charged galacturonic acid and form multi-layers at the surface of the oil droplets (Williams and Siew 2008, Siew et al. 2008). Many studies have shown protein as an important factor for emulsification but there is no simple relationship established (Siew et al. 2008, Williams et al. 2005). It could be different combinations of moieties producing different emulsification results (Siew et al. 2008). Emulsification properties are influenced by the accessibility of protein and ferulic acid to the surface of the oil droplets, proportion of ester groups and molecular mass distribution of the fractions (Williams et al. 2005). Larger MW fractions gave larger droplet sizes and unstable emulsions (Williams et al. 2005).

Ferulic acid is another hydrophobic portion present in the structure of SBP. In general, ferulic acid is found esterified to plant cell wall polysaccharides (Colquhoun et al. 1994) to C-2 of the arabinose residues and C-6 of the galactose residues in SBP (Colquhoun et al. 1994). About 30% of the feruloyl groups are carried by the arabinose side chains (Guillon and Thibault 1990).

Methyl ester groups and acetyl groups also provide hydrophobic character to pectin in general (Burapapadh et al. 2010, Dea and Madden 1986). Also, acetyl groups have been shown to prevent gel formation by SBP (Michel et al. 1985, Matora et al. 1995).

In this study, we used isopropanol fractionation to obtain fractions of sugar beet pectin and characterized each fraction for protein content, ferulic acid content, surface hydrophobicity, galacturonic acid content, particle size, zeta potential and degree of esterification. All fractions were structurally different and produced fractions rich in protein and ferulic acid and high surface hydrophobicity.

Materials and Methods

Materials

Sugar beet pectin- GENU® pectin type was obtained from CPKelco, Denmark (Batch no. GR91400), isopropanol was obtained from J.T Baker (Phillipsburg, NJ). The standards, ferulic acid (trans-4-hydroxy-3-methoxycinnamic acid) and D-galacturonic acid were obtained from Sigma Aldrich (St. Louis, MO). The fluorescent probe, 8-Anilino-1-naphthalene-sulfonic acid (ANS), was obtained from Invitrogen Molecular Probes (Eugene, OR). m-Hydroxydiphenyl was obtained from Eastman Kodak Company (Rochester, NY). Other chemicals were reagent grade and obtained from Fisher Chemical (Atlanta GA) or J.T Baker, (Phillipsburg, NJ).

Methods

Fractionation

A dispersion of 20 mg/mL SBP was made by slowly adding 20 g of SBP into 1000 mL of deionized Type II water. The dispersion was hydrated at 4 °C overnight. An aliquot of 100 mL SBP was removed as control and 100 mL isopropanol was added to the remaining 900 mL of the 20 mg/mL SBP dispersion. Isopropanol was added while pectin was mixed on a magnetic stir plate followed by centrifugation at 8,000xg, 4 °C for 20 min (Thermo Electron Corporation Sorvall RC 6 Plus High Speed Centrifuge (Ashville, NC). The supernatant was filtered using Miracloth™ (Calbiochem, La Jolla, CA). The pellet was spread evenly on a petri dish and placed in a fume hood to evaporate residual isopropanol. The dried pellet was ground using a Satake AC 100 grinder (Stafford, TX) and stored at 4 °C until analysis. Additional aliquots of 100 mL isopropanol were sequentially added to the supernatant obtained in the previous step, mixed, centrifuged, dried and stored as stated earlier, for a total of six fractionations. At the end of six fractionations, no further pellet was obtained and the amount obtained in Fraction 6 was variable. Fractions were denoted as F1, F2, F3, F4, F5 and F6 respectively. The initial dry powder or 100 mL control was used directly in subsequent analysis.

Protein assay

Protein contents of unfractionated SBP (control) and fractions F1-F6 were determined using the Pierce® BCA Protein Assay Kit (Bicinchoninic acid method) with bovine serum albumin as standard. The microplate was read at 560 nm using a BIO-RAD iMark microplate reader from Bio-Rad Laboratories (Hercules, CA).

Ferulic acid content

Ferulic acid was estimated at 310 nm using a Shimadzu UV-Pharmaspec 1700 UV-Visible spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). A standard curve between 0-12.5 µg ferulic acid was constructed using standard trans-ferulic acid (trans-4-hydroxy-3-methoxycinnamic acid) as described (Williams and Siew 2008). Dispersions of 0.05 mg AIS/mL SBP and SBP fractions in Type II water were used. The measurements were made at 310 nm.

Uronic acid assay

Dispersions of 0.1 mg AIS/mL and 0.2 mg AIS/mL of SBP and SBP fractions, respectively, were made and hydrated overnight at 4 °C. Uronic acid content was determined using the m-hydroxydiphenyl method (Asboe-Hansen and Blumenkrantz 1973). The sample absorbances at 520 nm were read between 2 and 5 min using a Thermo Electron Corporation SPECTRONIC 20D+ (West Palm Beach, FL).

Surface hydrophobicity

Fluorescence intensity was measured for all fractions and control at 2 mg AIS/mL and expressed as mg protein/mL. In addition, the surface hydrophobicity (S_0) of dispersions of SBP control and SBP fractions F1 and F4 was estimated by determining the slope of a regression analysis at protein concentrations between 0 and 400 µg protein/mL. Dispersions were hydrated overnight at 4 °C. An aliquot of 20 µL of 0.005 g/mL ANS stock, prepared daily, was added to 2 mL of sample. To prepare samples or ANS, Type I water was used. The blank was 2 mL of Type I water with 20 µL ANS and the intensity of the blank was subtracted from each sample reading. Fluorescence intensity measurements were made (SPEX FLUOROLOG Fluorometer,

Horiba Scientific, Edison, NJ) after 10 min equilibration using 60s time base acquisition with forward face geometry. Excitation and emission wavelengths were 380 nm and 475 nm, respectively; excitation and emission slits were 0.5 mm and 1.25 mm. The hydrophobic intensity was measured in cycles per second (cps) and corrected for daily lamp fluctuations relative to ethanol with ANS at the same acquisition conditions. A correction factor was calculated for each sample by taking the ratio of cps of the sample and cps of ethanol with ANS run on the same day and used to estimate Relative Fluorescence Intensity (RFI). The RFI was plotted against the five concentrations of the samples used. The slope (S_o) of the line for each sample was the surface hydrophobicity (Kato and Nakai 1980).

Particle size and ζ-potential

A 10 mM sodium phosphate buffer, pH 6.8, was made and filtered twice with a 0.45 µm cellulose nitrate (Whatman membrane filter, Piscataway, NJ). Dispersions of 0.75 mg AIS/mL of SBP and SBP fractions were prepared in sodium phosphate buffer at pH 6.8 (Kim and Wicker 2009). The samples were prepared in an ethanol sterilized, clean fume hood and hydrated overnight at 4 °C. The samples were filtered under the fume hood using a polyvinylidene (PVDF) resin Millex-AA syringe driven 5 µm Millipore membrane filter (Millipore, Billerica, MA). The filtered samples were transferred to deionized water rinsed cuvettes. The cuvette was capped and sonicated for 20 s (Fisher Scientific, Atlanta GA). A 90 Plus Particle Size Analyzer (Brookhaven Instruments Corporation, Holtsville, NY) was used to measure particle size using auto-slope normalization, dust filter ON, dust cut-off 85% for particle sizes greater than1000 nm and dust cut off 50% for particle sizes <1000 nm, 90% dust cut-off for fractions F1 and F2. After

the particle size was measured, ζ-potential was measured (BI-ZEL Electrode Assembly, SN:AQ-796).

FTIR

Powders of SBP and SBP fractions were tested on a Thermo Scientific Nicolet 600 FTIR from Thermo Scientific, West Palm Beach, FL. The settings used were ATR crystal, background measurement every 300 min and 64 scans per reading. The spectra were collected in the region of 4000-700 cm^{-1}. In this study, after baseline correction, peaks were normalized relative to a constant peak. The spectra of fractions F1-F6 and control were overlaid and normalized with respect to a peak. Each repeating peak was chosen and the peak heights were compared on the overlaying normalized spectra. The peak heights gave an idea about the concentration of the functional group represented by the chosen peak.

Experimental Statistical Analysis

An aliquot of 20 g sugar beet pectin was fractionated three times and the fractions obtained from each fractionation were characterized with the above techniques in triplicate. Averages of triplicate assays were reported. Minitab 16 software was used to perform ANOVA. Significant differences among the six fractions and control were thus estimated.

Results and Discussion

Fractionation and composition of fractions

With each 100 mL increment of isopropanol in the 20 mg SBP/mL sugar beet pectin dispersion, different yields of alcohol insoluble solids (AIS) were obtained (Table 3.1) and

fractions were visually different. No AIS was obtained after six increments. Pellet of F1 was dark grey, soft; F2 was off-white, soft and loose; F3 and F4 were transparent, gel-like and firm; F5 was gel-like and less firm; and F6 was white, grainy, very firm. The greatest AIS yield was obtained in F3 at 7,667 mg AIS/20 g SBP with the total addition of 300 mL isopropanol. The lowest yields of AIS were obtained in F1 and F6. Yield was highly variable between replications in some fractions. Pectic substances are typically precipitated by 50% ethanol and use of higher ethanol concentrations increased the co-precipitation of non-pectic alcohol insoluble ballast such as hemicelluloses consisting of arabans, galactan and xylans (Kertesz 1951). Use of propanol or acetone is less common and requires a lower percentage concentration to precipitate pectic substances. This is the first application of isopropanol precipitation and fractionation of pectic substances at propanol concentrations between about 10 and 60% propanol.

Protein assay

The concentrations of protein in the different fractions and the control are shown in Table 3.1. Fraction F1 had the highest concentration of protein whereas F4 had the lowest concentration. Fraction F4 was the only fraction with lower protein than unfractionated SBP control. Protein content in F1 and F2 was about twice the protein in unfractionated SBP. The range of values reported in literature for protein content of sugar beet pectin is from 2 to 10.4% (Kirby et al. 2008, Funami et al. 2011, Thibault 1988, Leroux et al. 2003, Williams et al. 2005, Mesbahi et al. 2005). The control sugar beet pectin in this study had 9% protein which is within the range of previously reported values.

Ferulic acid analysis

The ferulic acid contents of the fractions and control are shown in Table 3.1. Fraction F1 had the highest (93 µg/mg of AIS) and fraction F4 had the lowest (14 µg/mg of AIS) concentration of ferulic acid. Fraction F4 and F3 were the only fractions with less ferulic acid than unfractionated sugar beet pectin. Fractions F1 was nearly five times the ferulic acid in control SBP. Fractions F2-F6 and control had similar ferulic acid content.

Uronic acid assay

The uronic acid contents of fractions F1-F6 and control are shown in Table 3.1. Fraction F1 had the lowest uronic acid (UA) and fractions F2-F6 were not different than unfractionated SBP. The protein to UA and ferulic acid to UA ratio was noticeably higher in F1 compared to other fractions (Table 3.1).

Surface hydrophobicity

The relative fluorescence intensity (RFI) of each fraction and the control are shown in Table 3.1. The RFI was highest in F1, F2, and F3, which is about 50 times higher than the RFI determined in F4 and F5 (Table 3.1). RFI in F6 or control was negligible. RFI, measured at a constant mg AIS/mL was converted to RFI/µg protein since protein is likely to be the major contributor to surface hydrophobicity (S_0). In subsequent analysis, F1 and F4, representing extremes with respect to protein and ferulic acid content and control SBP were selected to estimate the surface hydrophobicity (Fig. 3.1). Surface hydrophobicity (S_o), estimated from the slope, was nearly 1.3 to 230 times higher in F1 than control or F4, respectively. Correlation

coefficients were ≥ 0.92. Fraction F1 had the highest slope (S_o), control SBP had an intermediate slope and fraction F4 had a negligible slope.

Particle size and ζ-potential

The particle sizes of different fractions are shown in Table 3.1. The control and earlier precipitating F1, F2 and F3 had higher particle size than later precipitating fractions F4, F5, and F6. Fraction F1 was the only fraction with greater particle size than unfractionated sugar beet pectin. The particle size of F1 was 0.4 times greater than unfractionated sugar beet pectin.

The range of ζ-potential values obtained for fractions F1-F6 and the control was -25 mV to -33 mV (Table 3.1). Despite the large differences in protein content and %DE in the fractions, the ζ-potential remained constant. If the ζ-potential is primarily attributed to the carboxylic acid group, then it is surprising that F1 with 11%DE was not different from F2-F6 or the control, suggesting that the protein moiety in F1 contributes to surface charge. In previous work (Kim and Wicker 2009) we reported that ζ-potential at pH 6.8 in dilute phosphate buffer, minimized some of the variation in ζ-potential of charge modified pectins associated with pH adjustment to 6.8 with NaOH, without altering the final ζ-potential of dilute dispersions of citrus pectin. The mean ζ-potential values in this study include some zero or no charge readings. In addition, fractions varied in viscosity at the concentration of 0.75 mg AIS/mL used in this study. While it is conceivable that protein, carboxylic acid, and overall structure contribute to surface charge, the potential contributions of diffusional differences are not separated in the measured ζ-potential values.

FTIR

The FTIR spectra for the protein rich F1, protein poor F4 and control SPB are depicted in Fig. 3.2a. The region from 1500-700 cm^{-1} is the fingerprint region for pectin (Chatjigakis et al. 1998). The spectra represents complex interacting vibrating systems and hence assignment of these bands to any particular vibrating system is labor intensive (Chatjigakis et al. 1998). Specifically, the peaks at 1319 cm^{-1}, 1231 cm^{-1}, 1015 cm^{-1} appear in the fingerprint region of sugar beet pectin. The peak at 1015 cm^{-1} appeared consistently in all SBP fractions, control SBP and citrus pectin (data not shown for citrus pectin). The 1015 cm^{-1} peak represents C-O stretching bonds and is a common peak associated with sugars and was thus chosen for normalization. Peak normalization did not allow comparison of peaks of different wavenumbers to each other but allowed comparison peaks of specific wavenumbers repeating in different fractions. The O-H stretching bands are sharp for free hydroxyl groups when the sample is in the vapor phase or a monolayer (3670-3580 cm^{-1}) (Socrates 2001). When concentration of the compound increases, and there is a lot of intermolecular hydrogen bonding present, the 3670-3580 cm^{-1} bands become broader and appear at lower frequencies (3580-3200 cm^{-1}). For solids, the broad band is usually centered around 3300 cm^{-1}. The OH peak in this case is not likely due to -COOH, since the -OH of –COOH actually causes further shifting to a lower wavenumber. Typically, it is a broad peak with a center at 3100 cm^{-1} with considerable amounts of carboxylic acid. The heights of the peak at 3300 cm^{-1} were not different between fractions and thus the concentration of –OH groups was not different in the fractions or control.

The band at ~3300 cm^{-1} was not attributed to ferulic acid either. The hydrogen bonding among OH groups that are on aromatic compounds absorb at 3580-3480 cm^{-1}. Also, phenol groups without bulky ortho groups have a broad absorption at 3400-3230 cm^{-1} and ferulic acid

does have -OCH_3 ortho group. The N-H stretching bands also occur in region 3350-3200 cm^{-1} when the sample is in the solid state. A slight shoulder was observed on the 3300 cm^{-1} broad peak, but there was no sharp peak in the expected N-H stretching region. So, no information about protein could be obtained from this region. It might be due to very little detectable protein or masking of the protein by water.

Information on the amide bond in proteins, formed between the carbonyl group and amine group of two amino acids can be obtained from the amide I band (C=O stretching) and amide II band (N-H bending) in an FTIR spectrum. The amide I bands for primary and secondary amides in the solid state lie in the region 1680-1630 cm^{-1}. No sharp bands were obtained in this region but a shoulder was obtained on another peak at 1620 cm^{-1}. In solid state, the primary amide is expected to show a weak to medium peak at 1650-1620 cm^{-1}, which is very close to the carbonyl band obtained at 1620 cm^{-1}. It was hard to resolve the shoulder into peaks. But amide II peak, which may represent secondary amides in the solid phase, was observed in 1570-1515 cm^{-1} region only for fractions F1 and F2 (Fig. 3.2a and Fig. 3.2b). Other fractions (Fig. 3.2a and Fig. 3.2b) showed a very weak shoulder in this region and no firm conclusions were possible. The presence of this peak supports the evidence from protein assay that fraction F1 and F2 were the most protein rich fractions.

Peaks at 1695-1540 cm^{-1} represent carboxylate groups in solution and the peak at ~1700 cm^{-1} represents carboxyl ester groups in solution. In the solid state, the absorptions 1600 cm^{-1} and 1700 cm^{-1} are shifted to a higher wavelength. This change caused by the physical state of the compound has been observed with glycine too (Costantino et al. 1997). The corresponding values of degree of esterification estimated from the peak heights are shown in Table 3.1. The degree of esterification of control SBP was 60%. The degree of esterification was lower than the

control in F1 and F2, about the same in F3 and higher than the control in F4, F5, and F6. The percent DE ranged from 11 to 74%.

FTIR was could not provide any particular information on acetyl groups. The acetyl group has multiple vibrations that are not unique only to the acetyl. It has the C=O stretching peak, around 1720 cm^{-1}, the CH_3 stretching peaks at 2960 cm^{-1} and 2870 cm^{-1}, the CH_3 deformations at 1460 cm^{-1} and 1375 cm^{-1}, C-CH_3 bending at 1370 cm^{-1}. All these peaks were present in the spectra, but they could be from the acetyl functional group or esters, aldehydes and other ketones, that are not acetyls. All these are common and present in food carbohydrates. A much larger study using controls and standards would be required to say which peaks were solely due to the acetyl groups. The peak at 1319 cm^{-1} was present very sharp in fraction F1 and F2. This peak may represent C-N stretching amide III band (1350-1310 cm^{-1}). FTIR was useful mainly to corroborate the evidence that fraction F1 was very different from other fractions and fractions F5 and F6 shared almost similar structural attributes.

While the emulsifying activity is typically attributed to greater amounts of protein, it may not necessarily mean better emulsifying properties (Funami et al. 2007, Siew et al. 2008). There is no simple relationship between protein and emulsifying activity. Factors like interference from the carbohydrate moiety (Castellani et al. 2010), and inaccessibility of the hydrophobic portion of the protein (Nakai 1983), may diminish the contribution of the protein to emulsification properties. Also, there are other hydrophobic groups like ferulic acid and acetyl groups that make it more difficult to establish relationships between emulsification capacity and hydrophobic groups. Molecular weights of the fractions play a bigger role to influence emulsification properties (Yapo et al. 2007b). Lower molecular weights are more conducive to emulsifying activity than gelling activity (Michel et al. 1985).

Conclusions

Isopropanol fractionation of SBP was selective and produced protein and ferulic acid rich fractions. Earlier precipitating fractions were richer in protein and ferulic acid and had higher particle size, lower %DE and greater fluorescence attributed to hydrophobicity than later precipitating fractions. Protein rich fractions were confirmed by FTIR analysis. The galacturonic acid content was lowest in the first fraction. Results confirm the heterogeneity of physico-chemical properties of SBP and provide information towards understanding the contribution to emulsifying activity of SBP.

References

Asboe-Hansen, G. & Blumenkrantz, N. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry, 54,* 484-489.

Burapapadh, K., Kumpugdee-Vollrath, M., Chantasart, D., & Sriamornsak, P. (2010). Fabrication of pectin-based nanoemulsions loaded with itraconazole for pharmaceutical application. *Carbohydrate Polymers, 82,* 384-393.

Castellani, O., Al-Assaf, S., Axelos, M., Phillips, G. O., & Anton, M. (2010). Hydrocolloids with emulsifying capacity. Part 2- Adsorption properties at the n-hexadecane-Water interface. *Food Hydrocolloids, 24,* 121-130.

Chatjigakis, A. K., Pappas, C., Proxenia, N., Kalantzi, O., Rodis, P., & Polissiou, M. (1998). FT-IR spectroscopic determination of the degree of esterification of cell wall pectins from stored peaches and correlation to textural changes. *Carbohydrate Polymers, 37,* 395-408.

Colquhoun, I. J., Ralet, M.-C., Thibault, J.-F., Faulds, C. B., & Williamson, G. (1994). Structure identification of feruloylated oligosaccharides from sugar-beet pulp by NMR spectroscopy. *Carbohydrate Research, 263,* 243-256.

Costantino, H. R., Griebenow, K., Langer, R., & Klibanov, A. M. (1997). On the pH memory lyophilized compounds containing protein functional groups. *Biotechnology and Bioengineering, 53,* 348-348.

Dea, I. C. M. & Madden, J. K. (1986). Acetylated pectic polysaccharides of sugar beet. *Food Hydrocolloids, 1,* 71-88.

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

Funami, T., Nakauma, M., Noda, S., Ishihara, S., Al-Assaf, S., & Phillips, G. O. (2008). Enhancement of the performance of sugar beet pectin as an emulsifier. *Foods Food Ingredients Journal, 213***,** 347-356.

Funami, T., Zhang, G., Hiroe, M., Noda, S., Nakauma, M., Asai, I., Cowman, M. K., Al-Assaf, S., & Phillips, G. O. (2007). Effects of proteinaceous moiety on the emulsifying properties of sugar beet pectin. *Food Hydrocolloids, 21***,** 1319-1329.

Guillon, F. & Thibault, J.-F. (1990). Oxidative cross-linking of chemically and enzymatically modified sugar-beet pectin. *Carbohydrate Polymers, 12***,** 353-374.

Kato, A. & Nakai, S. (1980). Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochimicha et Biophysica Acta., 624***,** 13-20.

Kertesz. (1951). *The pectic substances*. New York: Interscience Publishers.

Kim, Y. & Wicker, L. (2009). Valencia PME isozymes create charge modified pectins with distinct calcium sensitivity and rheological properties. *Food Hydrocolloids, 23***,** 957-963.

Kirby, A. R., MacDougall, A. J., & Morris, V. J. (2008). Atomic force microscopy of tomato and sugar beet pectin molecules. *Carbohydrate Polymers, 71***,** 640-647.

Leroux, J., Langendorff, V., Schick, G., Vaishnav, V., & Mazoyer, J. (2003). Emulsion stabilizing properties of pectin. *Food Hydrocolloids, 17***,** 455-462.

Matora, A. V., Korshunova, V. E., Shkodina, O. G., Zhemerichkin, D. A., Ptitchkina, N. M., & Morris, E. R. (1995). The application of bacterial enzymes for extraction of pectin from pumpkin and sugar beet. *Food Hydrocolloids, 9***,** 43-46.

Mesbahi, G., Jamalian, J., & Farahnaky, A. (2005). A comparative study on functional properties of beet and citrus pectins in food systems. *Food Hydrocolloids, 19***,** 731-738.

Michel, F., Thibault, J.-F., Mercier, C., Heitz, F., & Pouillaude, F. (1985). Extraction and characterization of pectins from sugar beet pulp. *Journal of Food Science, 50,* 1499-1502.

Nakai, S. (1983). Structure-function relationships of food proteins with an emphasis on the importance of protein hydrophobicity. *Journal of Agricultural and Food Chemistry, 31,* 676-683.

Siew, C. K., Williams, P. A., Cui, S. W., & Wang, Q. (2008). Characterization of the surface-active components of sugar beet pectin and the hydrodynamic thickness of the adsorbed pectin layer. *Journal of Agricultural and Food Chemistry, 56,* 8111-8120.

Socrates. (2001). *Infrared and Raman Characteristic Group Frequencies: Tables and Charts*. New York: John Wiley & Sons.

Thibault, J.-F. (1988). Characterization and oxidative crosslinking of sugar-beet pectins extracted from cossettes and pulps under different conditions. *Carbohydrate Polymers, 8,* 209-223.

Williams, P. A., Sayers, C., Viebke, C., & Senan, C. (2005). Elucidation of the emulsification properties of sugar beet pectin. *Journal of Agricultural and Food Chemistry, 53,* 3592-3597.

Williams, P. A. & Siew, C. K. (2008). Role of protein and ferulic acid in the emulsification properties of sugar beet pectin. *Journal of Agricultural and Food Chemistry, 56,* 4164-4171.

Yapo, B. M., Robert, C., Etienne, I., Wathelet, B., & Paquot, M. (2007a). Effect of extraction conditions on the yield, purity and surface properties of sugar beet pulp pectin extracts. *Food Chemistry, 100,* 1356-1364.

Yapo, B. M., Wathelet, B., & Paquot, M. (2007b). Comparison of alcohol precipitation and membrane filtration effects on sugar beet pulp pectin chemical features and surface properties. *Food Hydrocolloids, 21***,** 245-255.

Table 3.1
Structural Characterization of Sugar Beet Pectin (SBP) SBP fractions

Fraction	Weight of fraction obtained (mg/20g)	Protein (µg/mg AIS)	Ferulic acid (µg/mg AIS)	GalA (µg/mg AIS)	RFI/µg protein	Particle size (nm)	ζ (mV)	DE (%)	Protein: GalA	Ferulic acid:GalA
1	$693^{c} \pm 137$	$209^{a} \pm 13$	$93^{a} \pm 20$	$220^{b} \pm 17$	$254^{a} \pm 68$	$1539^{a} \pm 124$	$-31^{a} \pm 5$	$11^{d} \pm 2$	0.95	0.42
2	$1190^{c} \pm 981$	$154^{b} \pm 56$	$26^{b} \pm 1$	$353^{a} \pm 53$	$260^{a} \pm 176$	$1298^{b} \pm 177$	$-33^{a} \pm 1$	$46^{c} \pm 7$	0.44	0.07
3	$7667^{a} \pm 1164$	$106^{cd} \pm 8$	$18^{b} \pm 4$	$385^{a} \pm 31$	$159^{ab} \pm 21$	$1091^{b} \pm 35$	$-33^{a} \pm 3$	$62^{b} \pm 3$	0.27	0.05
4	$3773^{b} \pm 757$	$77^{d} \pm 9$	$14^{b} \pm 6$	$408^{a} \pm 55$	$5^{b} \pm 0$	$395^{cd} \pm 125$	$-31^{a} \pm 3$	$71^{a} \pm 2$	0.19	0.03
5	$1570^{c} \pm 223$	$88^{cd} \pm 19$	$19^{b} \pm 5$	$379^{a} \pm 42$	$2^{b} \pm 0$	$278^{d} \pm 201$	$-25^{a} \pm 14$	$74^{a} \pm 2$	0.23	0.05
6	$437^{c} \pm 212$	$122^{bc} \pm 43$	$23^{b} \pm 7$	$349^{a} \pm 45$	$0.003^{ab} \pm 0$	$680^{c} \pm 176$	$-29^{a} \pm 2$	$70^{a} \pm 2$	0.35	0.07
Control	-	$87^{cd} \pm 5$	$19^{b} \pm 2$	$352^{a} \pm 22$	$0.01^{ab} \pm 0$	$1241^{b} \pm 38$	$-33^{a} \pm 0$	$60^{b} \pm 0$	0.25	0.05

(Means that do not share a letter are significantly different at p=0.05)

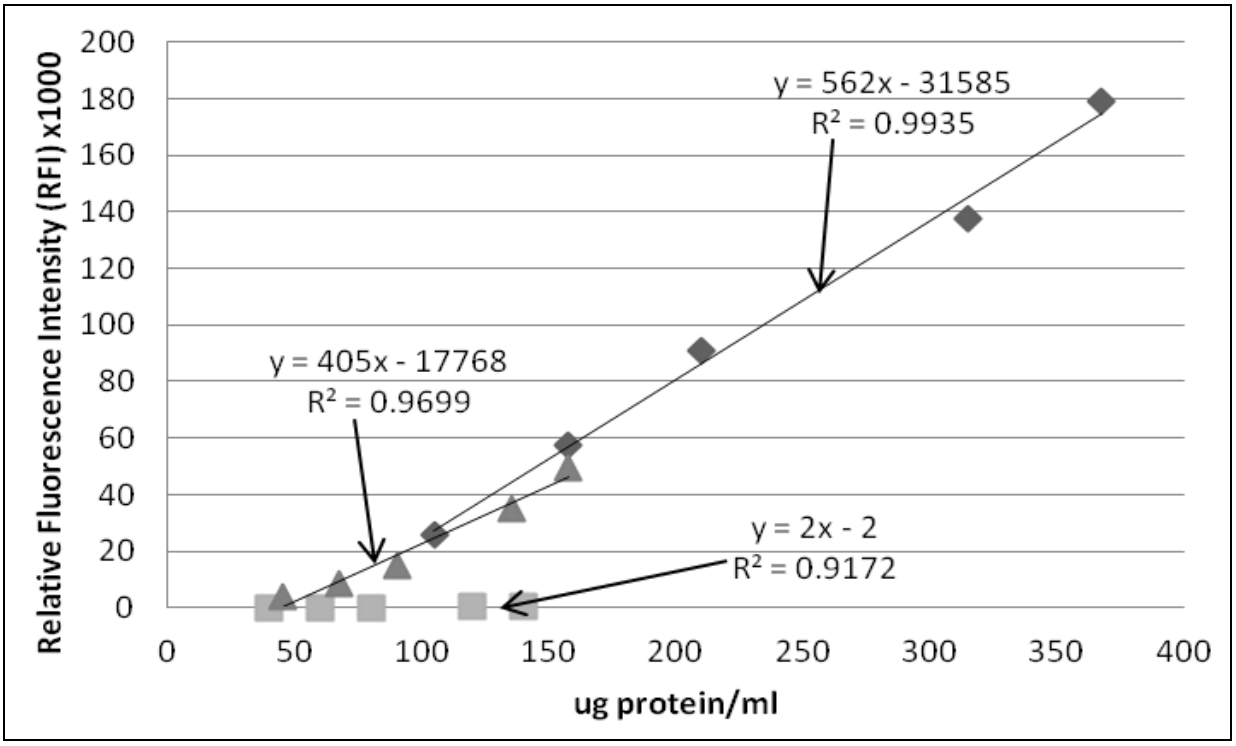

Fig. 3.1. The relative fluorescence intensity (RFI) values of Fractions F1, F4 and control plotted against the concentration. F1 ◆; F4 ; Control ▲

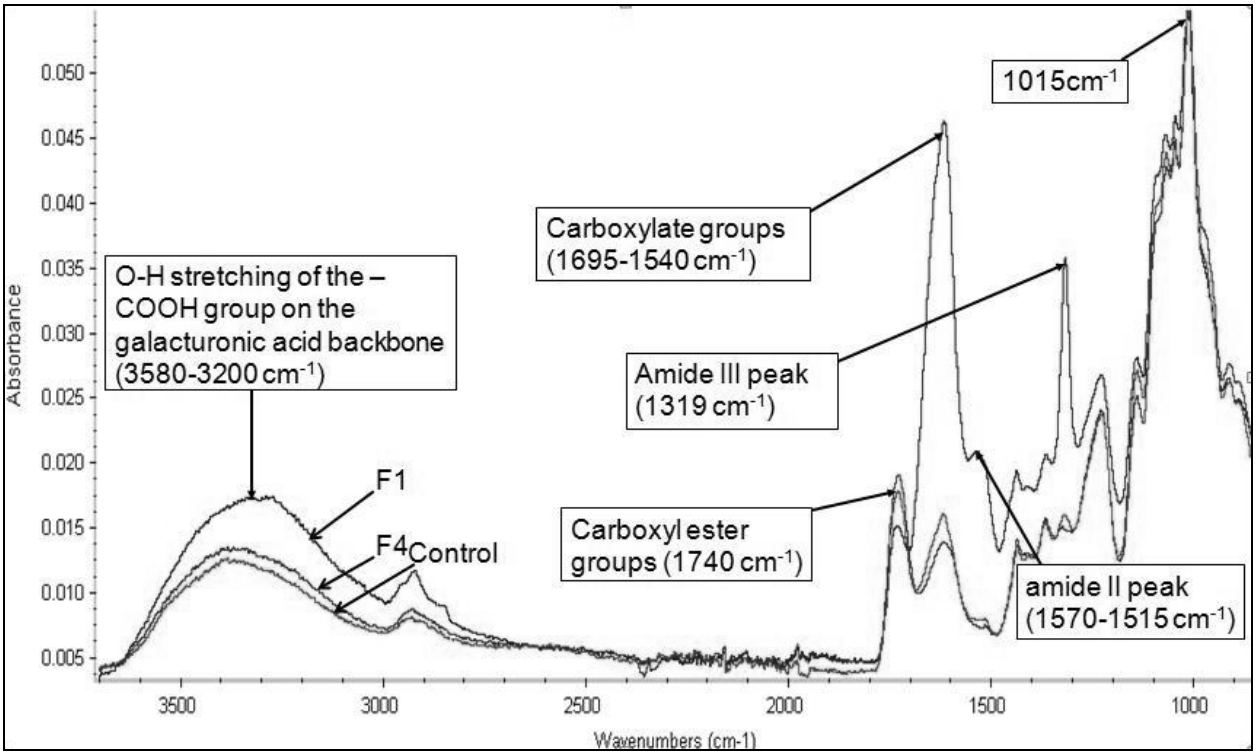

Fig. 3.2a. Fourier Transform Infrared (FTIR) spectra for Fractions F1, F4 and control.

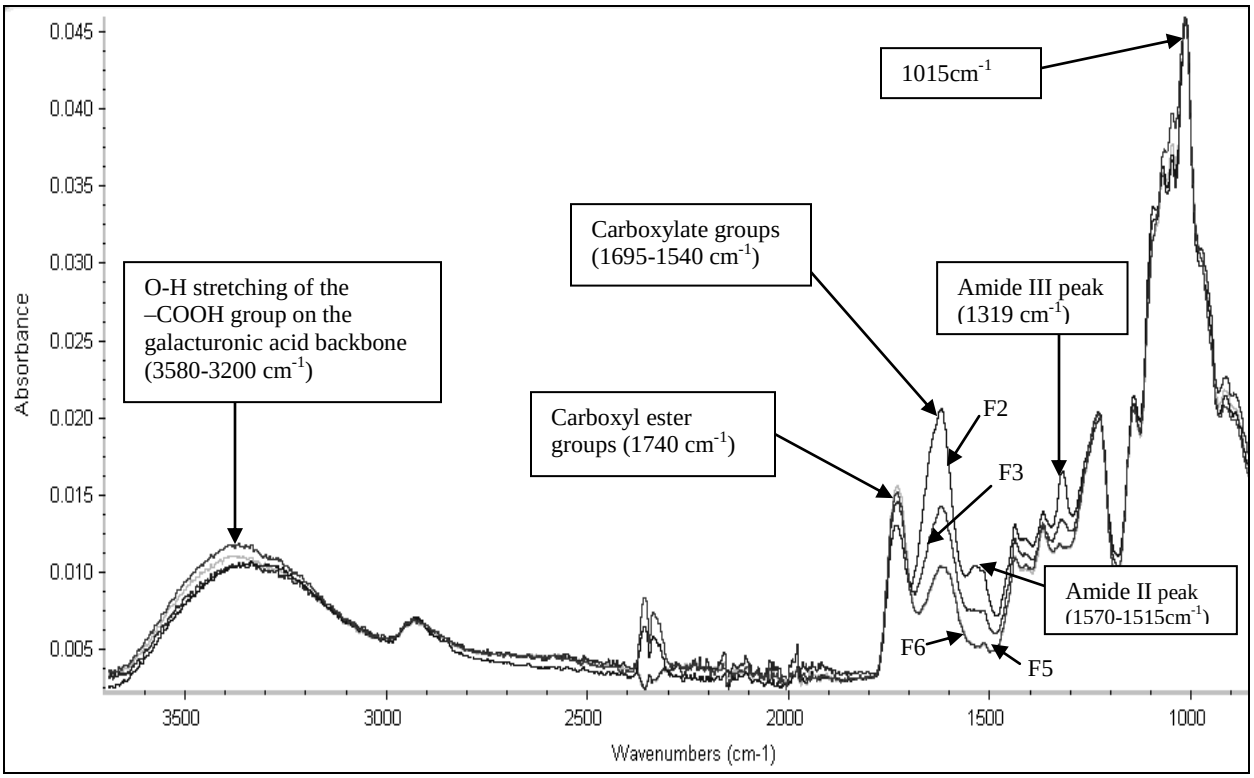

Fig. 3.2b.Fourier transform infrared (FTIR) spectra for Fractions F2, F3, F5 and F6.

CHAPTER 4

EMULSION STABILITY OF SUGAR BEET PECTIN FRACTIONS OBTAINED BY ISOPROPANOL FRACTIONATION[2]

[2] Karnik, D. and Wicker, L. To be submitted to Food Hydrocolloids.

Abstract

Isopropanol fractionation was used to obtain protein rich (F1) and protein poor (F4) fractions of sugar beet pectin (SBP). It was hypothesized that the protein rich fraction with greater surface hydrophobicity would have superior emulsification capacity. Different concentrations (1.5% and 0.5%) of F1 and F4 each were used with 15% (medium chain triglyceride) MCT oil to make oil-in-water emulsions. The control emulsion was made using 1.5% unfractionated SBP. The emulsions were maintained over ice throughout preparation time and passed 3 times under high pressure homogenization at 35MPa. They were stored at 32ºC for 10 days. The D[4,3] values for all emulsions were below 1.5µm. Fraction F4 showed the lowest droplet sizes at 1.5% concentration and was less by 0.16µm than the control. Also, F4 showed better emulsification capacity and stability at 0.5% than the protein rich fraction at 0.5% (Emulsion F1.0.5 had D[4,3], D[3,2] and span values higher by 0.45µm, 0.21µm and 0.52, respectively than the control and Emulsion F4.0.5 had D[4,3], D[3,2] and span values higher by 0.1µm, 0.07µm and 0.15, respectively than the control). The reason for better performance of the protein poor fraction may have been because of poor accessibility of the protein in the protein rich fraction or the carbohydrate moiety playing a role in making the protein bulky, decreasing packing parameter, increasing curvature of the droplets and hence decreasing droplet size. It was confirmed that there was no simple relationship between protein, ferulic acid content and emulsification capacity. Dark field microscopy was able to validate the results obtained from laser diffraction and there were no significant changes in viscosities of individual emulsions over 10 days.

INDEX WORDS: Sugar beet pectin, Emulsions, MCT oil, Droplet size, Dark field microscopy, Rheology.

Introduction

The food industry uses pectin from apple pomace, citrus peels and sugar beet as the major sources. Sugar beet pectin (SBP) is very different from the apple and citrus pectin because of the presence of higher amounts of hydrophobic acetyl groups (Michel et al. 1985) on the galacturonic acid chain, more number of hairy regions, more phenolic esters of ferulic acid and higher amount of protein covalently linked (Kirby et al. 2008) to the hairy regions. The fraction of sugar beet pectin adsorbed on the oil phase of an emulsion contains most of the hydrophobic groups like protein (Williams and Siew 2008, Siew et al. 2008, Leroux et al. 2003, Akhtar et al. 2002, Funami et al. 2007) and ferulic acid (Williams and Siew 2008, Siew et al. 2008, Funami et al. 2007). Also, the ratio of galacturonic acid to side chains is low in the adsorbed pectin fraction (Williams and Siew 2008). It is proposed that the positively charged protein moieties interact with the negatively charged galacturonic acid and form multi-layers at the surface of the oil droplets (Siew et al. 2008, Williams and Siew 2008). Many studies have shown protein as an important factor for emulsification but there is no simple relationship established (Siew et al. 2008, Williams et al. 2005). It could be different combinations of moieties producing different emulsification results (Siew et al. 2008). Emulsification properties are influenced by the accessibility of protein and ferulic acid to the surface of the oil droplets, proportion of ester groups and molecular mass distribution of the fractions (Williams et al. 2005). Larger MW fractions (306 kDa-562 kDa) gave larger droplet sizes and less stable emulsions (Williams et al. 2005). The higher amount of acetyl groups are believed to confer the emulsifying properties instead of gelling (Leroux et al. 2003), although, treatment with hydrogen peroxide/peroxidase and ammonium persulfate causes gelling in SBP (Oosterveld et al. 1997). SBP is also characterized by very low molecular weight (Michel et al. 1985).

The proposed mechanism by which SBP acts as an emulsifying agent is that the hydrophobic moieties like protein adsorb and anchor on to the oil droplets and reduce the interfacial tension between the oil and water interface (Funami et al. 2007, Leroux et al. 2003). The carbohydrate moiety stabilizes by steric and viscosity effects in the aqueous phase (Leroux et al. 2003). SBP has been looked upon as an alternative to gum arabic, a very efficient food emulsifying agent because gum arabic has comparatively very little protein and thus has to be used in large quantities (~15%). SBP has more protein than gum arabic and citrus pectin and can be used in smaller quantities (~1.5% - 3%). Also, SBP has an extended configuration and a semi-flexible chain with radius of gyration of 39nm whereas gum arabic has a globular, coil configuration with radius of gyration of 28nm (Castellani et al. 2010). So, SBP can cover the oil droplets better than gum arabic, at the same concentration. In other words, 2% SBP can produce the same reduction in interfacial tension as 15% gum arabic (Akhtar et al. 2002, Leroux et al. 2003).

SBP was enhanced using a patented maturation process. The process was performed by heating sugar beet pectin powder at 50 to 150 °C at a relative humidity of 20 to 90% for 1 to 48 hours. This enhancement caused the proteins to aggregate and thus form a bulky carbohydrate layer around the oil droplets (Funami et al. 2008). The bulky carbohydrate provided good steric stabilization and it was difficult to disaggregate the proteins. This prevented flocculation for a long time and improved emulsion stability. The process also produced orange oil and lime oil emulsions that were stable to thermal sterilization, unlike the untreated control SBP. Medium chain triglycerides (MCT) have a fatty acid chain length between 6 to 10 carbon atoms. They are more compatible with sugar beet pectin than limonene oil (Funami et al. 2008).

Sugar beet pectin has been subjected to fractionation in a previous study by (Williams et al. 2005). They used hydrophobic affinity chromatography for fractionation. All the fractions differed in protein, ferulic acid and molecular weight. The low molecular weight fractions were able to make emulsions with smaller droplet sizes and were stable for a longer time as compared to the high molecular weight fractions. The relationship between molecular weight and droplet size of emulsions has been verified (Yapo et al. 2007b). Williams et al. (2005) could not draw a firm conclusion about the relationship between protein and ferulic acid content and emulsification property. Funami et al. (2007) made emulsions with 15% MCT oil and 1.5% SBP both unmodified and modified with proteinases. The modified SBP with reduced protein content, molecular weight and radius of gyration gave emulsion with increased droplet size (from 0.56 μm to 3 μm). It also caused creaming and had much less amount than usual pectin fraction, adsorbed on the oil phase. When Funami et al. (2011) used SBP (both non-enhanced and enhanced) to cleave protein, polygalacturonic backbone and side chains separately, they observed that the loss of protein caused the maximum loss of emulsification capacity, followed by the side chains and the backbone cleavage. Cleaving the side chains reduced both the protein content and the ferulic acid content. Also, the protease and arabinase/galactase cleaved SBP produced emulsions with larger droplet sizes and lower creaming stability. These results indicated that the protein moiety in SBP contributed significantly to the emulsifying property. SBP was also fractionated using isopropanol to obtain protein and ferulic acid rich and poor fractions (Karnik, Hawkins and Wicker 2012).

In this study, we hypothesize that protein and ferulic acid rich fractions would be able to produce emulsions with smaller droplet sizes because of the hydrophobic groups. Also, a change

in the concentration of emulsifier should produce different droplet sizes and different consistencies.

Materials and Methods

Materials

Sugar beet pectin- GENU® pectin type BETA was donated by CPKelco (Copenhagen, Denmark, Batch no. GR91400). Isopropanol and sodium benzoate were obtained from J.T Baker (Phillipsburg, NJ). Medium chain triglyceride was obtained from (Now Sports MCT oil from Now Foods, Bloomingdale, IL.)

Methods

Fractionation

A dispersion of 20 mg/mL SBP was made by slowly adding 60 g of SBP into 3000 mL of deionized (DI) Type II water. The dispersion was stirred at 4 °C overnight. An aliquot of 300 mL isopropanol was added to 2700 mL of the 20 mg/mL SBP dispersion. The isopropanol was allowed to mix well for 10 min and then the mixture was centrifuged at 8,000 x g, 4 °C for 20 min using Thermo Electron Corporation Sorvall RC 6 Plus High Speed Centrifuge from Thermo Scientific, Ashville, NC. The supernatant was separated from the pellet by filtration using a filter cloth from Miracloth™, (Calbiochem, La Jolla, CA). The pellet was spread evenly on a Petri dish and allowed to dry under a fume hood to evaporate residual isopropanol. The dried pellet was ground using a Satake AC 100 grinder from Stafford, TX and stored at 4 °C. An aliquot of 300 mL isopropanol was added to the supernatant obtained in step 1, mixed for 10 min, centrifuged and stored as described earlier. This process was repeated until 4 fractions were

obtained. These alcohol insoluble solids (AIS) collected at successive alcohol additions were denoted as Fractions F1, F2, F3 and F4, respectively. Based on preliminary data, in addition to the control, Fractions F1 and F4 were selected for further analysis because these fractions were protein and ferulic acid rich and poor, respectively. Results from three replications were reported.

Emulsion

Five different emulsions were prepared using fractions F1, F4 and unfractionated control SBP and the concentrations of the respective emulsifiers were varied as 0.5% or 1.5%. The other parameters were kept constant. The five different emulsions were labeled F1.1.5, F1.0.5, F4.1.5, F4.0.5 and C. Sugar beet pectin (1.5 g or 0.5g) was dissolved in 80 g DI water. The solution was hydrated and stirred overnight at 4 °C. The SBP solution was homogenized at speed 4 for 2 min at 4 °C using a PRO Scientific Inc. PRO300A homogenizer. Solution of 10% sodium benzoate was made by dissolving 5 g sodium benzoate in 50 mL type II deionized water. An aliquot of 1 g of the 6300 mol/L sodium benzoate solution and 15 g MCT oil was added to the homogenized solution. The remaining weight was made up to 100 g using DI water. The mixture was homogenized again at 24,000 rpm for 2min. This was the crude emulsion. The crude emulsion was immediately homogenized at 35 MPa (Avestin EmulsiFlex-C5 from Avestin Inc., Ottawa, Canada) for a total of three passes; emulsions were collected on ice between passes.. The compositions of the five emulsions are shown in Table 1. The emulsions were stored in glass jars with screw caps at 32 °C (THELCO Model 4, Thermo Scientific, Ashville, NC) in an accelerated shelf life test.

Emulsion characterization

The particle size was measured using a Malvern Mastersizer 2000 (Worcestershire, UK). The emulsion was diluted in the dispersion unit by stirring at 2000 rpm. It was added drop by drop to the dispersion unit till an obscuration value between 10-30% was obtained. The droplet diameters were reported on a volume basis D[4,3] and surface area basis D[3,2]. The relative span value $(d_{90} - d_{10})/d_{50}$ (calculated by the software)was used to report the distribution width of the emulsions. The droplet size and distribution width were measured each day of storage.

Steady stress controlled tests were done on the emulsions on day 0 and day 10 of storage using a Rheometric Scientific SR-5000 stress rheometer (Thermo Scientific, Ashville, NC) with couette geometry. The gap between the cylinder and cup was 0.5 mm. An aliquot of 20 mL of sample was kept on ice for an hour to lower its temperature to 4 °C, and was filled into the cup and allowed to relax for 5 min with the cylinder inside the cup (Meriem-Benziane et al. 2012). The sample was then subjected to steady stress sweep test. The range of stress applied on the sample was 0.1 Pa to 100 Pa. The rheological parameters tested were viscosity, yield stress and consistency.

Dark field microscopy was done on the emulsions on days 0, 5 and 10. The sample was put on a glass slide and covered gently with a coverslip. It was viewed with 100X oil immersion lens. Spot software was used to make image settings and a camera was connected to the microscope. The image settings used in the Spot software were dark field-transmitted light, brightness 1.0-1.5, gain limit 16, gamma 0.25-0.4, correct color technology, tone- neutral, resolution- normal, chip imaging area- full chip, display full chip area in live mode, noise filter- on, flatfield 100.

Experimental statistical analysis

An aliquot of 60 g SBP was fractionated in triplicate and from each of the three fractionations, Fractions 1 and 4 were used to make emulsions in triplicate. Each replication of the emulsions was tested for droplet size and dark field microscopy in triplicate. The average of triplicate assays was reported. The rheological parameters were tested once for each replication of the emulsions because of insufficient sample for measurements in triplicate. Using Minitab 16 software, unpaired t test was used to compare significant differences between the initial droplet sizes of emulsions when the concentration of emulsifier was varied. Analysis of variance (ANOVA) and Tukey test was used to determine if initial droplet sizes were significantly different for all five emulsions. Unpaired t test was also used to determine significant changes in the rheological parameters of the emulsions on day 0 and day 10 of storage.

Results and Discussion

Droplet size

On day 0, all emulsions had D[4,3] values less than 1.5µm and D[3,2] less than 1µm. The range of D[4,3] values for emulsions were (0.67 µm-1.42 µm). This particle size range is very close to the range observed in previous studies on SBP emulsions. The values observed were about 5 µm with limonene oil (Williams and Siew 2008); about 0.71 µm with 15% MCT oil (Funami et al. 2007); about 0.64 µm with 15% MCT oil (Funami et al. 2008), 0.4-0.5 µm with 20 % orange oil (Leroux et al. 2003).

Smaller droplet diameters on day 0 are considered to be better for stability of an emulsion and a narrow distribution width indicates a good emulsion with uniformly sized droplets. Increase in the distribution width indicates unfavorable changes in the emulsions. Relative span

is one of the ways to represent distribution width. Relative span is given by $(d_{90} - d_{10})/d_{50,}$ where d_{90} is the diameter below which 90% of the droplets can be placed, d_{10} the diameter below which 10% of the droplets can be placed, and d_{50} the diameter below which 50% of the droplets can be placed. The changes in D[4,3] and D[3,2] values for the emulsions are shown in Fig 4.1a and 4.1b, respectively.

Behavior of F1 and F4 at 1.5% concentration

On day 0, emulsion F4.1.5 gave the smallest D [4,3], D [3,2] and span values and these were less by 0.16µm, 0.13µm and 0.22 respectively, than the control emulsion (Emulsion C) on day 0. Emulsion F1.1.5 had D[4,3], D[3,2] and span values higher by 0.59µm, 0.23µm and 1.1 , respectively, than the control. Also, in the microscopic image, the droplets of F4.1.5 looked uniform sized, with no flocculation and good dispersion (Fig.4.4C). This confirmed the result from laser diffraction which showed one sharp narrow peak. Whereas, for F1.1.5, the droplets did not seem to have very uniform size and were not evenly dispersed. Flocculation was observed (Fig.4.4A). Thus at 1.5% concentration, the protein poor fraction was more effective. Also, both F1 and F4 did not have constant droplet sizes over 10 days.

Behavior of F1 and F4 at 0.5% concentration

On day 0, emulsion F1.0.5 had D[4,3], D[3,2] and span values higher by 0.45µm, 0.21µm and 0.52 respectively than the control and Emulsion F4.0.5 had D[4,3], D[3,2] and span values higher by 0.1µm, 0.07µm and 0.15 respectively than the control. Though, as observed by dark field microscopy, the droplet sizes of Emulsion F4.0.5 looked bigger (Fig.4.4D) than those of Emulsion F4.1.5 (Fig.4.4C). The droplet size looked uniform but there was slight flocculation

observed, confirmed by the slight shoulder on the distribution peak. Again, at 0.5% concentration, the protein poor fraction was better than the protein rich fraction. At 0.5%, the emulsions prepared with the protein and ferulic acid rich fraction (F1) did not show smaller particle size and the particle size grew more with time as evidenced by the higher span value.

Also, emulsion F1.0.5 did not maintain emulsion stability because the protein, although higher in concentration may not have been accessible (Williams et al. 2005) and the high protein:pectin ratio (0.95) in Fraction F1 suggests that there was not enough carbohydrate for steric stabilization (Siew et al. 2008), as compared to the protein poor fraction (protein:pectin ratio of 0.19). Emulsion F4.0.5 showed small droplet sizes because the protein was probably enough to cover the droplets and showed greater stability probably because of a higher proportion of carbohydrate moiety as compared to the protein rich fraction.

Therefore, a similar trend was observed at both the concentrations (0.5% and 1.5%) for both the fractions (F1 and F4). This confirmed that there was no simple relationship between protein and emulsification properties (Siew et al. 2008, Williams and Siew 2008). Also, different concentrations of emulsifier or different oil-SBP ratios produced different droplet sizes and were important factors in determining emulsification capacity.

Analysis of observed behavior

In previous work (Karnik et al. 2012- in preparation), particle size varied between fractions of SBP in that fractions F1, F4 and control had particle sizes of 1539nm, 395nm, and 1241nm respectively and may have been a factor for their observed behavior in oil-in-water emulsions. Polymers with a larger particle size are less flexible and do not quickly coat the droplet surface (Dickinson 2003). The protein rich emulsifier had large particle size (Karnik et

al. 2012- in preparation) and was not readily soluble as compared to the protein poor fraction. The large particle size may have slowed down the rate of coating the oil droplets during high pressure homogenization and thus reduced efficiency for emulsification.

Also, the protein and ferulic acid rich fractions used in this study had greater surface hydrophobicity (Karnik et al. 2012- in preparation) but did not result in better emulsification capacity as measured by droplet size and stability during storage. The reason why the protein rich fraction showed poor performance than the protein poor fraction at 1.5% concentration may be due to the poor accessibility of protein in the protein rich fraction (Williams et al. 2005). Also, there might be strong inter-molecular association of protein moieties on pectin chains in the protein rich fraction, thus hindering emulsification activity. Markman and Livney et al (2012) found that the conjugating casein protein with oligosaccharides made the hydrophilic portions of the protein bulky and decreased their packing parameter. This increased the droplet curvature of the encapsulated hydrophobic bioactive compound and gave smaller size of encapsulated droplets. Also, the saccharide layer formed a protective outer shell for stabilization. This mechanism may be playing a role in better emulsifying properties associated with low protein-high carbohydrate SBP fractions. The protein poor fraction had greater proportion of hydrophilic groups and thus might have a higher hydrophilic-lipophilic balance (HLB). Also since the MCT oil is readily miscible in water, the higher proportion of hydrophilic groups must be more compatible with the MCT oil, leading to better performance of F4 with MCT oil.

Based on initial droplet size and span values, emulsions prepared with C were superior to Emulsions F1.1.5, F1.0.5 and F4.0.5. Emulsions F1.1.5 and F1.0.5, not only showed a broad span on day 0, but also showed a small second peak on the droplet size distribution on day 0 (Fig. 4.2a and 4.2b). On day 10 their distribution was broader and an additional peak was

observed (Fig. 4.2c and 4.2d). The additional peak observed for pectin emulsions was seen to disappear on addition of a surfactant like Tween 20 (Akhtar et al. 2002). A competitive displacement of pectin by Tween 20 on the oil-water interface was proposed. Thus the additional peak on the droplet size distribution for SBP emulsions may have appeared because of flocculation of oil droplets. Dilution and surfactant methods (Akhtar et al. 2002) need to be performed in order to confirm that flocculation was a major reason for instability in emulsions made in this study. The flocculation was observed in dark field microscopy (discussed later). If the suspected flocculation was reversible, it may have been depletion flocculation, where the concentration of emulsifier used must have been more than sufficient. The excess emulsifier failed to bind to the oil droplets, causing it to deplete from the oil surface and form weak, reversible bonds between the oil droplets.

Fraction F1 was not completely soluble in water unlike fraction F4. The solubility was not quantified in this study but was visually confirmed. The insoluble material sedimented after sitting for 5 minutes. Also it was more difficult to filter a dispersion of fraction F1 than to filter dispersions of other fractions with no insoluble material. This behavior was not like pectin and there may have been some insoluble non-pectin material interfering with emulsifying activity.

On day 0, Emulsion F4.1.5 showed D[4,3], D[3,2] and span smaller by 0.26µm, 0.2µm and 0.37, respectively than emulsion F4.0.5. These values for emulsion F4.1.5 were significantly lower than that of emulsion F4.0.5 on day 0 ($p<0.05$). Although Emulsion F4.1.5 showed smallest droplet size on day 0, Emulsion F4.0.5 was the most stable over 10 days of storage at 32°C. All emulsions except F4.0.5 showed successive increase in droplet sizes up to about 4 µm from day 0 to day 10. Overall, the Emulsion F4.0.5 had smaller droplet size that changed the least during storage.

Rheology

Since oil-in-water emulsions like salad dressings have non-Newtonian behavior (Zhang et al. 2008), rheology was used to characterize some non-Newtonian properties like shear thinning or shear thickening behavior, viscosity and also to determine if the emulsions have yield stress. The tests were carried out under steady state shear stress. Viscosity may be used as a quality control reference (Zhang et al. 2008) and changes in viscosity over a period of time may indicate loss of quality (McClements 2007).

Steady state flow curves of 'viscosity and shear rate' and 'shear stress and shear rate' were obtained in triplicate for each emulsion. Fig. 4.3 shows the plots of emulsion C on day 0. The 'viscosity and shear rate' plots were fit into the Power Law model and the shear stress and shear rate plots were fit into the Casson model and Herschel-Bulkley model. The measurements were made on day 0 and day 10 of storage. The power law model provided information about the consistency and shear thinning or shear thickening behavior of the emulsions. Since the emulsions were able to flow very freely and the 'viscosity and shear rate' curves showed a pseudoplastic behavior (shear thinning behavior) or dilatant behavior (shear thickening behavior), it was concluded that the emulsions did not have yield stress (i.e. they were not viscoplastic).

Emulsions showed significant differences ($p<0.05$) between each other in viscosity and consistency, but the viscosity, and consistency for an individual emulsion did not change significantly ($p>0.05$) from day 0 to day 10. The viscosity, and consistency values of the emulsions are shown in Table 4.2. Emulsions F1.1.5, F4.1.5 and C had shear thinning behavior on both day 0 and day 10 of storage, whereas emulsions F1.0.5 and F4.0.5 had shear thickening

behavior on day 0 and day 10. Usually food emulsions show shear thinning behavior. Shear thinning occurs because of separation of flocculated droplets at high shear rates or droplets getting aligned to the flow field (McClements 2007). Shear thinning is accompanied by decrease in viscosity. Shear thickening occurs when there is flocculation and the droplets do not separate at high shear rates because they are tightly packed (McClements 2007). Emulsions F1.0.5 may have shown shear thickening because the oils droplets may have had stronger forces of attraction between the flocculated oil droplets at the lower concentration (0.5%) of emulsifier F1. So, at high shear rates, they were not able to separate. So, the droplets were tightly packed and thus showed shear thickening throughout the storage.

Microscopy

Dark field microscopy depicted emulsions on days 0, 5 and 10 (Fig. 4.4). In the microscopic image of Emulsion F1.1.5, (Fig. 4.4A) the droplets did not seem to have very uniform size and were not evenly dispersed. Flocculation was observed, consistent with particle size, laser diffraction results. On day 5, larger droplets could be observed. Groups of droplets appeared close together instead of being dispersed. This confirms the suspected flocculation which was observed in the droplet size distribution of the emulsion. Thus protein and ferulic acid rich fractions had poor emulsification capacity, as indicated by laser diffraction analysis. Flocculation was observed, as indicated by laser diffraction, but the emulsion did not appear as broken as emulsion F1.0.5 (Fig. 4.4B)

In the microscopic image of emulsion F1.0.5, the droplets seemed to have more uniform sizes relative to Emulsion F1.1.5. Flocculation could be observed, which was also indicated by the extra peak on the droplet size distribution of laser diffraction. These observations further

confirmed that protein and ferulic acid rich fraction had poor emulsification capacity. Fraction F1 at 0.5% concentration looked more uniform in droplet size but the dispersion did not look good. In conclusion, protein and ferulic acid rich fractions did not show very good emulsification capacity. On day 5, larger droplets were observed. Groups of droplets appeared close together instead of being dispersed. This confirmed the flocculation observed in the droplet size distribution of the emulsion. On day 10, emulsion F1.0.5 showed a greater increase in mean droplet size than emulsion F1.1.5.

On day 0, from laser diffraction, Emulsion F1.0.5 had a mean D [4,3] value of 1.2µm. The span was not very narrow and there were droplets smaller and greater than 1.2µm present. A third peak was observed on day 10 (Fig. 4.2c and 4.2d) by laser diffraction and the span value broadened a lot more than emulsion F1.1.5. Accordingly, Emulsion F1.0.5 looked more broken than Emulsion F1.1.5 on day 10. In conclusion, at lower concentration, protein and ferulic acid rich fraction showed better emulsification capacity, but was not able to maintain the emulsion stability.

On day 0, Emulsion F4.1.5 had 0.67µm mean droplet size and narrow span according to laser diffraction. In the microscopic image, the droplets looked uniform sized, with no flocculation and good dispersion. This confirmed the result from laser diffraction which showed one sharp narrow peak. On day 5, droplet size looked bigger and flocculation was observed. This was also in agreement to the laser diffraction results. On day 10, flocculation was observed. The droplet size looked much bigger than day 0. The dispersion no longer looked uniform like on day 0.

On day 0, the droplet sizes of Emulsion F4.0.5 looked bigger than those of Emulsion F4.1.5. The droplet size looked uniform but there was slight flocculation observed, confirmed by the slight shoulder on the distribution peak. On day 5, a second peak was observed on the droplet

size distribution. The dark field image did not show a visible change in the emulsion droplet size and the dispersion but flocs of oil droplets sticking together were observed. On day 10, flocculation was observed. The droplets looked bigger than observed on day 0. Emulsion F4.1.5 showed bigger, more prominent flocs on day 10 than Emulsion F4.0.5.

The dark field microscope image was mainly used to visualize and confirm dispersion, coalescence or flocculation and droplet size uniformity of the emulsion instead of determining the actual droplet sizes. Dark field microscopy is mainly used to observe outlines of unstained objects and is not ideal to determine actual sizes or study internal structure of the objects. The different background colors obtained in some images were due to small differences in the thickness of the mount or presence of some insoluble artifacts in the aqueous medium of the emulsions.

Conclusions

Results indicated that SBP and fractions formed oil-in-water emulsions that varied in emulsification capacity and stability that was not directly related to protein or ferulic acid content. Ratio of SBP fraction to oil influenced emulsification capacity and stability. At 0.5% concentration, protein and ferulic acid rich fraction showed better emulsification capacity, but was not able to maintain the emulsion stability. Change in concentration of the emulsifier changed the behavior of the emulsions under shear stress.

References

Akhtar, M., Dickinson, E., Mazoyer, J., & Langendorff, V. (2002). Emulsion stabilizing properties of depolmerized pectin. *Food Hydrocolloids, 16,* 249-256.

Castellani, O., Al-Assaf, S., Axelos, M., Phillips, G. O., & Anton, M. (2010). Hydrocolloids with emulsifying capacity. Part 2- adsorption properties at the n-hexadecane-water interface. *Food Hydrocolloids, 24,* 121-130.

Dickinson, E. (2003). Hydrocolloids at interfaces and the influence on the properties of dispersed systems. *Food Hydrocolloids, 17,* 25-39.

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

Funami, T., Nakauma, M., Noda, S., Ishihara, S., Al-Assaf, S., & Phillips, G. O. (2008). Enhancement of the performance of sugar beet pectin as an emulsifier. *Foods Food Ingredients Journal, 213,* 347-356.

Funami, T., Zhang, G., Hiroe, M., Noda, S., Nakauma, M., Asai, I., Cowman, M. K., Al-Assaf, S., & Phillips, G. O. (2007). Effects of proteinaceous moiety on the emulsifying properties of sugar beet pectin. *Food Hydrocolloids, 21,* 1319-1329.

Karnik, D (2012). Fractionation of sugar beet pectin using isopropanol and characterization of the fractions obtained. Athens: University of Georgia.

Kirby, A. R., MacDougall, A. J., & Morris, V. J. (2008). Atomic force microscopy of tomato and sugar beet pectin molecules. *Carbohydrate Polymers, 71,* 640-647.

Leroux, J., Langendorff, V., Schick, G., Vaishnav, V., & Mazoyer, J. (2003). Emulsion stabilizing properties of pectin. *Food Hydrocolloids, 17,* 455-462.

Markman, G. & Livney, Y. D. (2012). Maillard-conjugate based core-shell co-assemblies for nanoencapsulation of hydrophobic nutraceuticals in clear beverages. *Food and Function, 3*, 262.

McClements, D. J. (2007). Critical review of techniques and methodologies for characterization of emulsion stability. *Critical Reviews in Food Science and Nutrition, 47,* 611-649.

McClements, D. J. *Food Emulsions: Principles, Practices and Techniques* (2004). *CRC Press.*

Meriem-Benziane, M., Abdul-Wahab, S. A., Benaicha, M., & Belhadri, M. (2012). Investigating the rheological properties of light crude oil and the characteristics of its emulsions in order to improve pipeline flow. *Fuel, 95*, 97-107.

Michel, F., Thibault, J.-F., Mercier, C., Heitz, F., & Pouillaude, F. (1985). Extraction and characterization of pectins from sugar beet pulp. *Journal of Food Science, 50,* 1499-1502.

Oosterveld, A., Grabber, J. H., Beldman, G., Ralph, J., & Voragen, A. G. J. (1997). Formation of ferulic acid dehydrodimers through oxidative cross-linking of sugar beet pectin. *Carbohydrate Research, 300*, 179-181.

Siew, C. K., Williams, P. A., Cui, S. W., & Wang, Q. (2008). Characterization of the surface-active Components of sugar beet pectin and the hydrodynamic thickness of the adsorbed pectin layer. *Journal of Agricultural and Food Chemistry, 56,* 8111-8120.

Williams, P. A., Sayers, C., Viebke, C., & Senan, C. (2005). Elucidation of the emulsification properties of sugar beet pectin. *Journal of Agricultural and Food Chemistry, 53*, 3592-3597.

Williams, P. A. & Siew, C. K. (2008). Role of protein and ferulic acid in the emulsification properties of sugar beet pectin. *Journal of Agricultural and Food Chemistry, 56,* 4164-4171.

Yapo, B. M., Wathelet, B., & Paquot, M. (2007). Comparison of alcohol precipitation and membrane filtration effects on sugar beet pulp pectin chemical features and surface properties. *Food Hydrocolloids, 21*, 245-255.

Zhang, C., Quek, S. Y., Lam, G., & Easteal, A. J. (2008). The rheological behaviour of low fat soy-based salad dressing. *International Journal of Food Science and Technology, 43*, 2204-2212.

Table 4.1. Formulation of the emulsions.

Emulsion	Emulsifier used	Weight of Emulsifier (g)	Weight of DI water type II (g)	Hydrated while stirring overnight at 4°C	Homogenized at 24,000 rpm for 2 minutes at 4°C	Weight of sodium Benzoate (g)	Weight of MCT oil (g)	Weight of water to make up to 100g (g)	Homogenized at 24,000 rpm for 2 minutes at 4°C	Number of passes at 35MPa
F1.1.5	F1	1.5	80			1	15	2.5		3
F1.0.5	F1	0.5	80			1	15	3.5		3
F4.1.5	F4	1.5	80			1	15	2.5		3
F4.0.5	F4	0.5	80			1	15	3.5		3
C	C	1.5	80			1	15	2.5		3

Table 4.2. Rheological parameters of emulsions

	Emulsion F1.1.5		Emulsion F1.0.5		Emulsion F4.1.5		Emulsion F4.0.5		Emulsion C1.5	
	Day 0	**Day10**	**Day 0**	**Day10**	**Day 0**	**Day10**	**Day 0**	**Day10**	**Day 0**	**Day10**
Viscosity (mPa-s)	24^{b}±1	27^{b}±1	8^{c}±0	8^{c}±0	64^{a}±6	72^{a}±10	12^{c}±0	11^{c}±2	65^{a}±0	70^{a}±13
Consistency	54±4	63±5	5±0	4±0	104±20	108±21	11±1	13±6	129±2	143±41
Power Law Index (PLI)	0.871^{d}± 0.008	0.862^{d}± 0.009	1.1^{a}± 0.02	1.12^{a}± 0.01	0.92^{c}± 0.02	0.93^{c}± 0.02	1.03^{b}± 0.02	0.97^{b}± 0.08	0.88^{cd}± 0.0	0.88^{cd}± 0.03
Behavior	Shear thinning	Shear thinning	Shear thickening	Shear thickening	Shear thinning	Shear thinning	Shear thickening	Shear thickening	Shear thinning	Shear thinning

1.All values are represented as mean ± standard deviation.
2.Consistency is expressed as multiplied by 10^{3}.
3. Means that do not share a letter are significantly different at p=0.05

Table 4.3. Droplet size distributions of emulsions expressed as span

F1.1.5		F1.0.5		F4.1.5		F4.0.5		C	
Day	**Droplet size distribution width(span)**	**Day**	**Droplet size distribution width(span)**	**Day**	**Droplet size distribution width(span)**	**Day**	**Droplet size distribution width(span)**	**Day**	**Droplet size distribution width(span)**
0	1.75	**0**	1.17	**0**	0.43	**0**	0.80	**0**	0.65
1	nd	**1**	2.57	**1**	0.54	**1**	0.81	**1**	2.29
2	2.68	**2**	2.44	**2**	Nd	**2**	0.82	**2**	2.13
3	2.43	**3**	2.37	**3**	0.71	**3**	0.81	**3**	1.93
4	2.67	**4**	2.32	**4**	0.76	**4**	Nd	**4**	nd
5	2.58	**5**	Nd	**5**	0.89	**5**	0.97	**5**	1.78
6	2.63	**6**	2.30	**6**	Nd	**6**	0.82	**6**	1.72
7	2.61	**7**	2.65	**7**	4.14	**7**	0.84	**7**	1.69
8	nd	**8**	Nd	**8**	4.71	**8**	0.84	**8**	1.62
9	2.71	**9**	Nd	**9**	6.53	**9**	0.88	**9**	1.66
10	2.50	**10**	3.33	**10**	3.26	**10**	0.83	**10**	1.62

nd= not determined

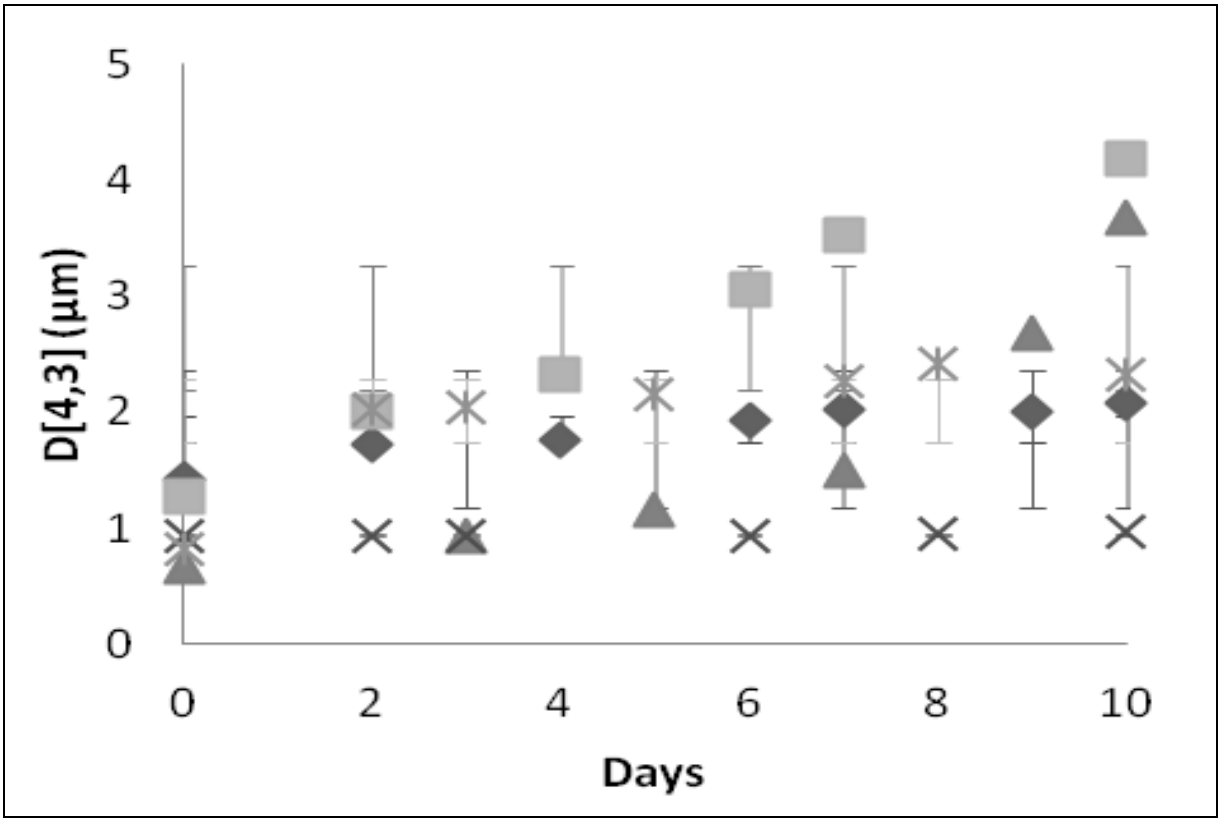

Fig. 4.1a. Changes in D[4,3] of all the emulsions over 10 days at 32°C. ◆ Emulsion F1.1.5; ■ Emulsion F1.0.5; ▲ Emulsion F4.1.5; X Emulsion F4.0.5; ✳ Emulsion C1.5. The D[4,3] values of Emulsions F1.1.5, F1.0.5, F4.1.5, F4.0.5 and C on day 0 were1.42µm, 1.28µm, 0.67µm, 0.93µm and 0.83µm respectively and the D[4,3] values of emulsions F1.1.5, F1.0.5, F4.1.5, F4.0.5 and C increased by 0.67 µm, 2.9 µm, 3.0 µm, 0.05 µm and 1.5 µm respectively, after 10 days storage at 32°C.

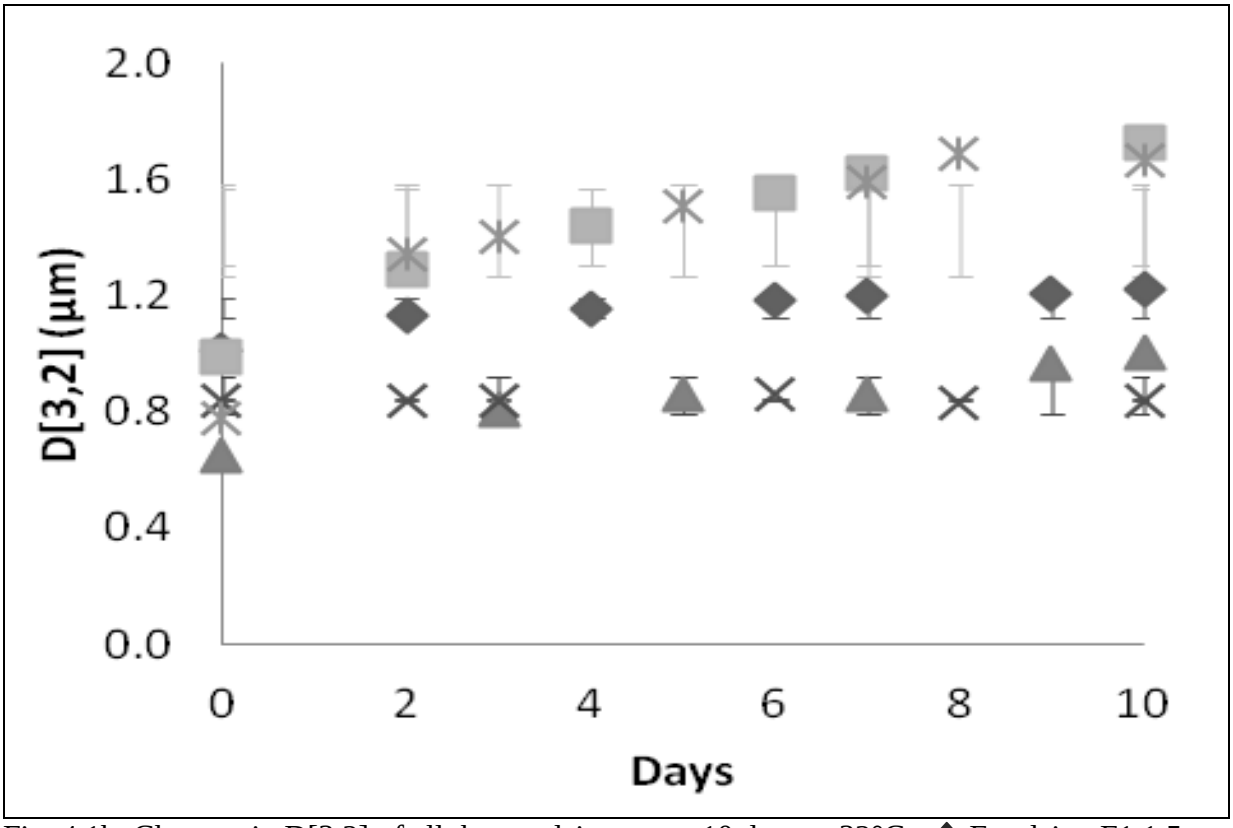

Fig. 4.1b. Changes in D[3,2] of all the emulsions over 10 days at 32°C . ◆ Emulsion F1.1.5; ■ Emulsion F1.0.5; ▲ Emulsion F4.1.5; X Emulsion F4.0.5; ✳ Emulsion C1.5. The D[3,2] values of the emulsions on day 0 were 1.01µm, 0.99µm, 0.65µm, 0.85µm and 0.78µm respectively and the D[3,2] values of the emulsions increased by 0.21µm, 0.74µm, 0.35µm, 0.01µm and 0.89µm respectively, after 10 days storage at 32°C.

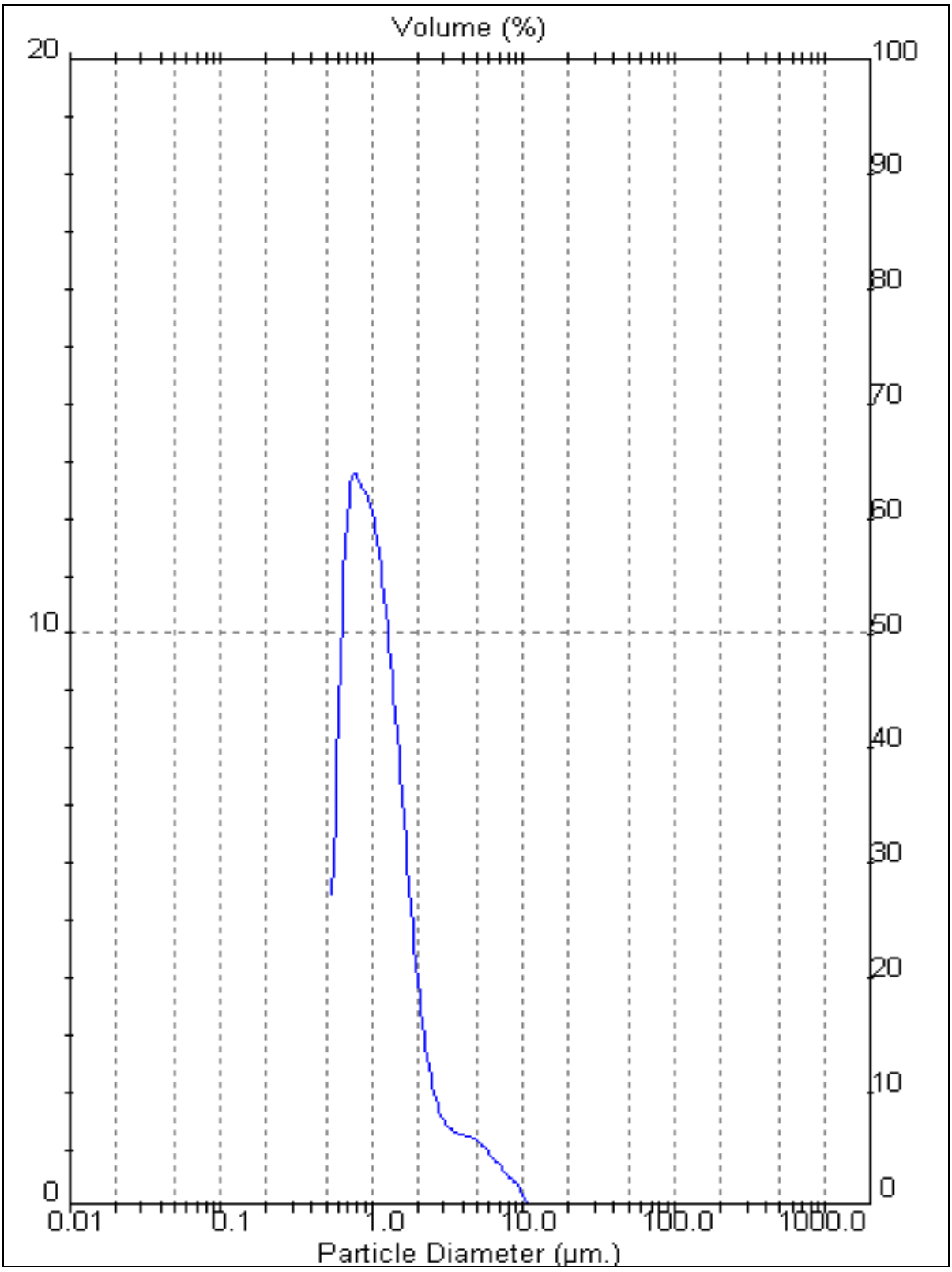

Fig 4.2a. Droplet size distribution of emulsion F1.1.5 on day 0.

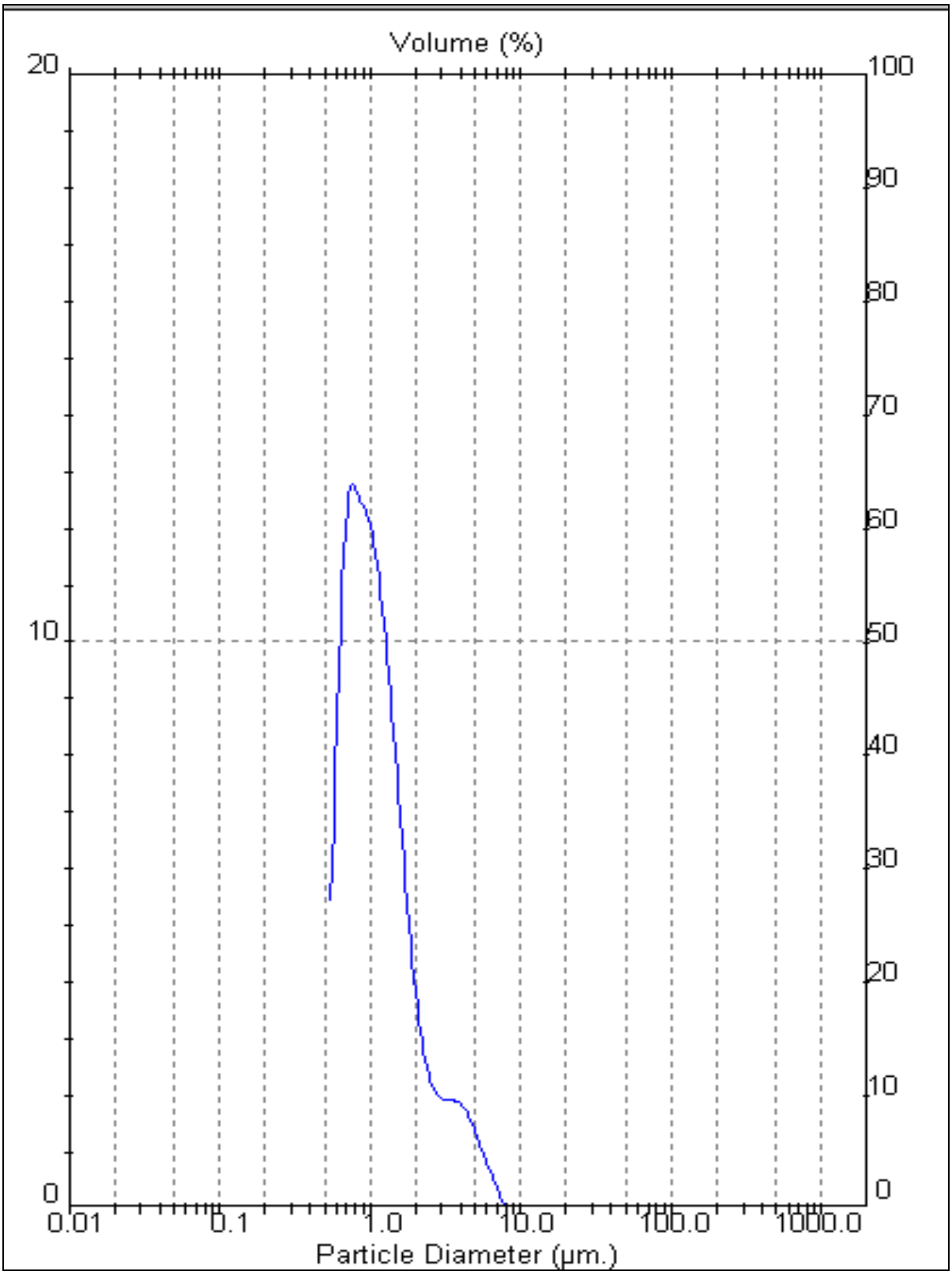

Fig 4.2b. Droplet size distribution of emulsion F1.0.5 on day 0.

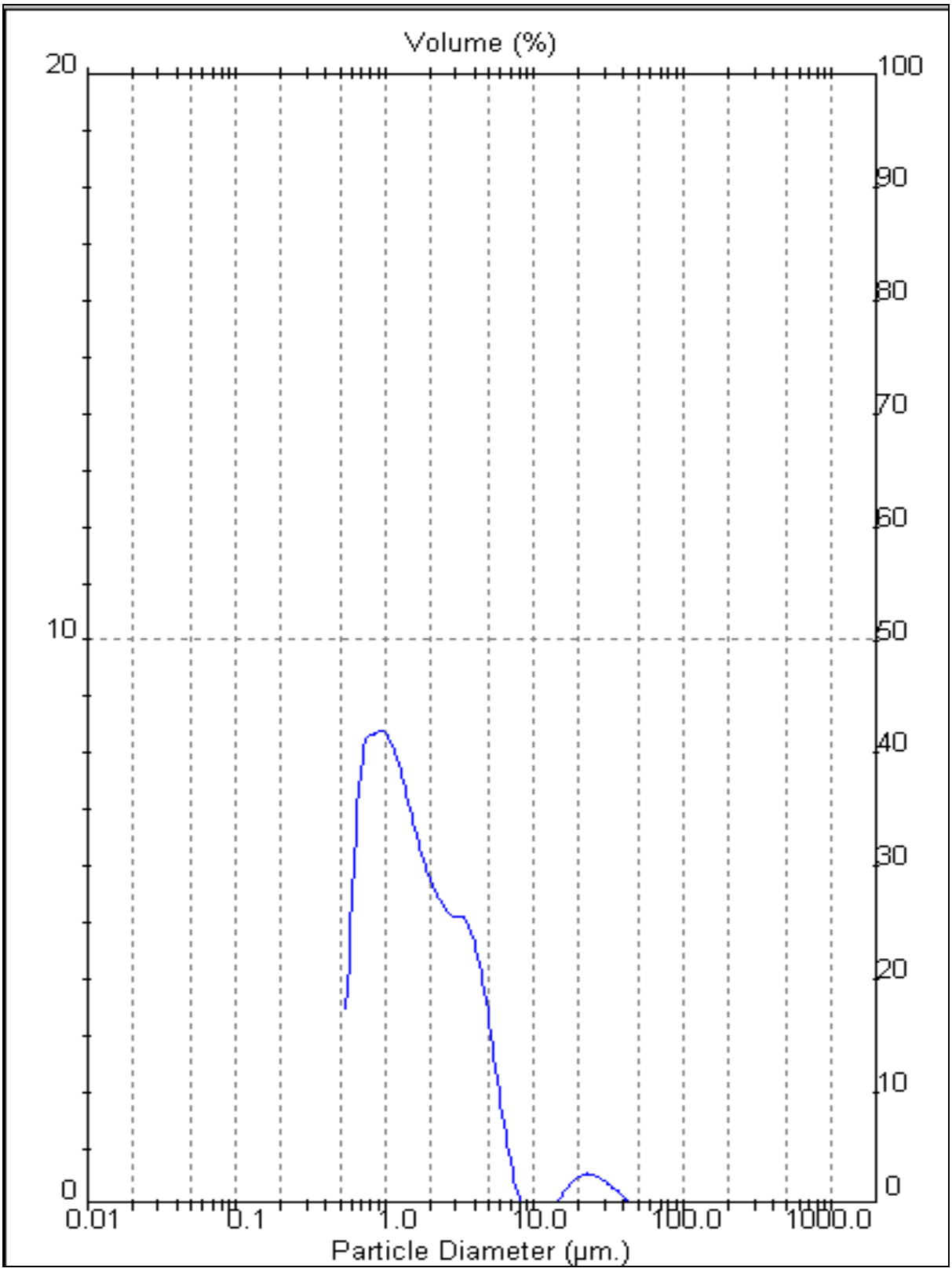

Fig 4.2c. Droplet size distribution of emulsion F1.1.5 on day 10.

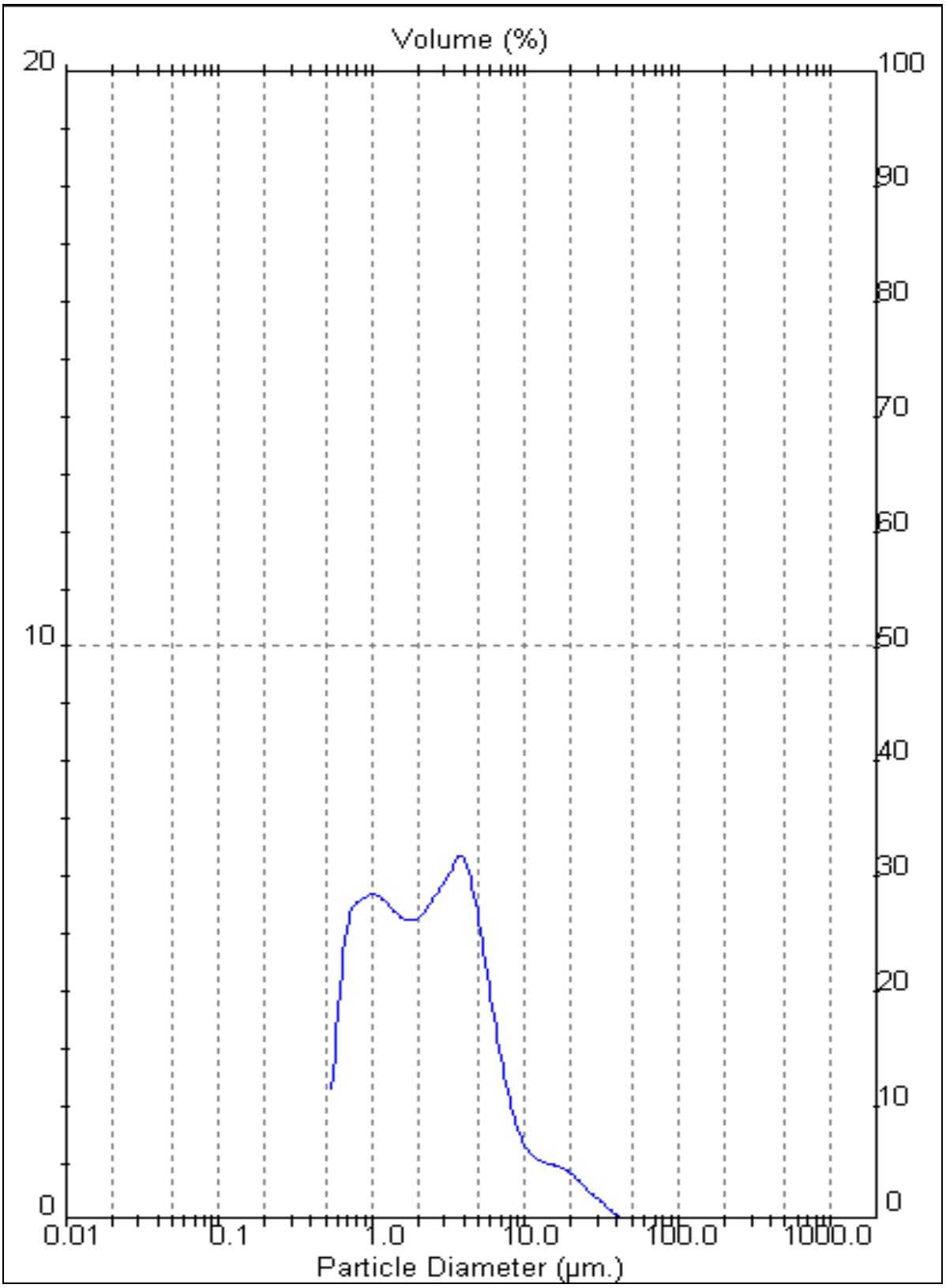

Fig 4.2d. Droplet size distribution of emulsion F1.0.5 on day 10.

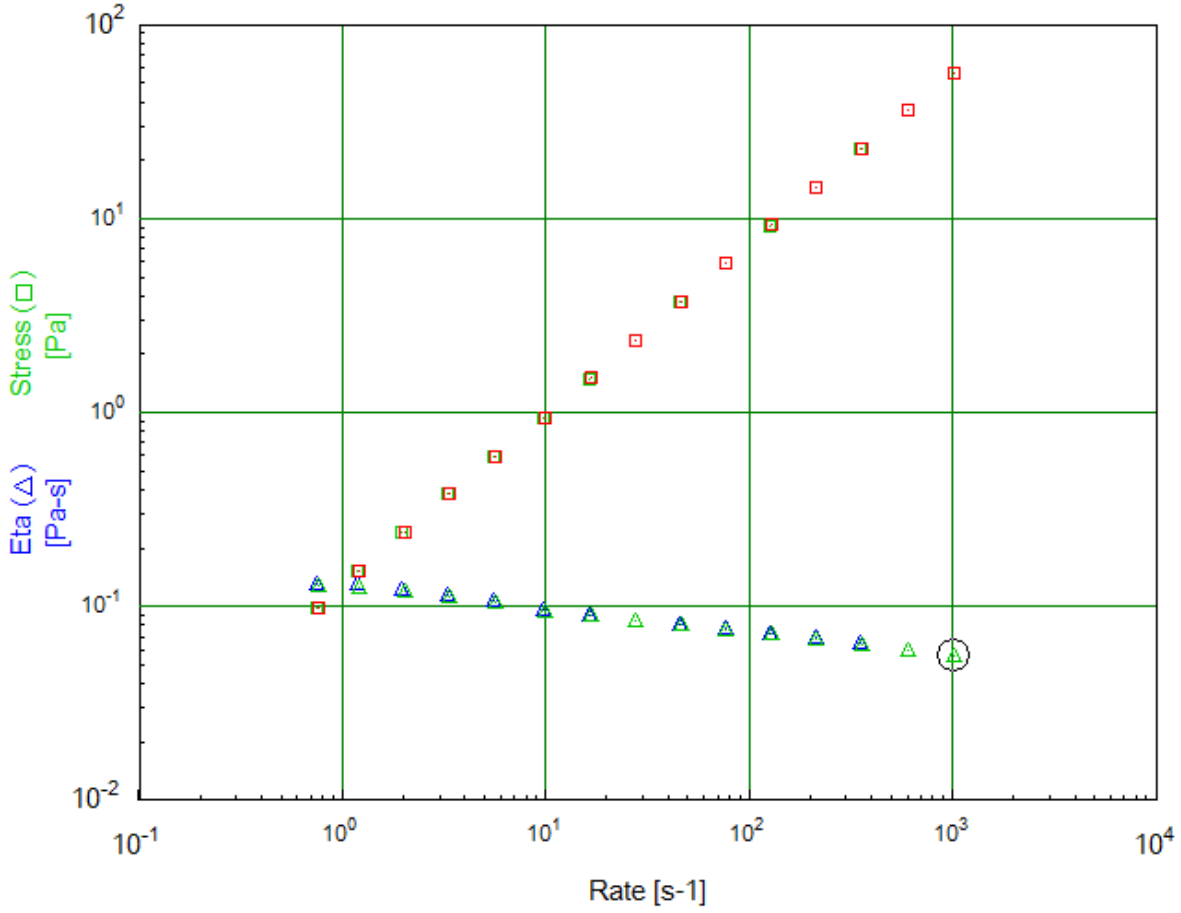

Fig. 4.3. Rheological behavior of emulsion C under steady state increasing shear rate on day 0.

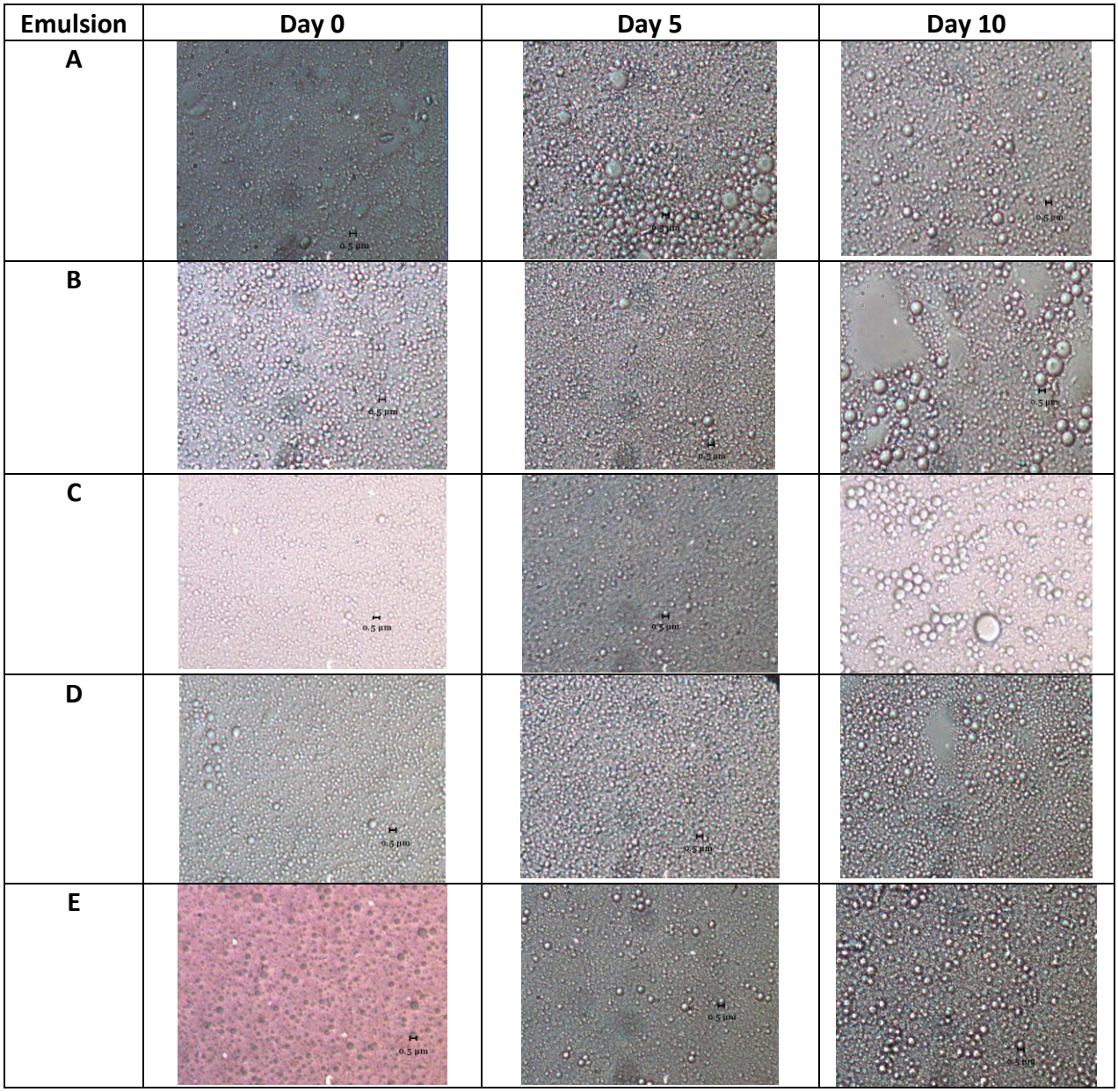

Fig. 4.4. Dark field microscopy images of Emulsions F1.1.5(A), F1.0.5(B), F4.1.5(C), F4.0.5(D), C(E) on day0, day5 and day10 of storage at 32°C.

CHAPTER 5

CONCLUSIONS

It was interesting to fractionate sugar beet pectin (SBP) and study the behavior (emulsion capacity and stability) with medium chain triglyceride (MCT) oil having potential health benefits, because SBP can be used in smaller amounts as compared to other emulsifying hydrocolloids to provide similar emulsification capacity. SBP with the health promoting oil would have applications in foods like salad dressings if SBP fractions showed good emulsification capacity as well as good stability. Isopropanol fractionation of SBP was able to yield fractions with high and low surface hydrophobicities. This method was promising for having structurally tailor-made SBP fractions. Protein, ferulic acid and acetyl groups may have collectively contributed to surface hydrophobicity. The protein poor fraction (less hydrophobic fraction) showed overall better stability as compared to protein rich fraction and unfractionated SBP. Thus, this study suggested that low protein-high carbohydrate SBP fraction may play a role for better stability. Also it was confirmed that there was no simple relationship between surface hydrophobicity and emulsification capacity for the isopropanol fractionated SBP fractions.