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ENCAPSULATING FATTY ACID ESTERS OF BIOACTIVE COMPOUNDS IN STARCH

A Dissertation in
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by
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ABSTRACT

Interest in the use of many bioactive compounds in foods is growing in large part because of the apparent health benefits of these molecules. However, many of these compounds can be easily degraded during processing, storage, or their passage through the gastrointestinal tract before reaching the target site. In addition, they can be bitter, acrid, or astringent, which may negatively affect the sensory properties of the product. Encapsulation of these molecules may increase their stability during processing, storage, and in the gastrointestinal tract, while providing controlled release properties.

The ability of amylose to form inclusion complexes and spherulites while entrapping certain compounds has been suggested as a potential method for encapsulation of certain molecules. However, complex formation and spherulitic crystallization are greatly affected by the type of inclusion molecules, type of starch, and processing conditions. The objectives of the present investigation were to: a) study the effect of amylose, amylopectin, and intermediate material on spherulite formation and its microstructure; b) investigate the formation of amylose and high amylose starch inclusion complexes with ascorbyl palmitate, retinyl palmitate, and phytosterol esters; c) evaluate the ability of spherulites to form in the presence of fatty acid esters and to entrap ascorbyl palmitate, retinyl palmitate, and phytosterol esters; and d) evaluate the effect of processing conditions on spherulite formation and fatty acid ester entrapment.

Higher ratios of linear to branched molecules resulted in the formation of more and rounder spherulites with higher heat stability. In addition to the presence of branches, it appears that spherulitic crystallization is also affected by other factors, such as degree of branching, chain length, and chain length distribution.

Amylose and Hylon VII starch formed inclusion complexes with fatty acid esters of ascorbic acid, retinol, or phytosterols. However, only retinyl palmitate formed a complex with amylopectin. In general, ascorbyl palmitate resulted in the highest
complexation, followed by retinyl palmitate and phytosterol ester. The presence of native lipids in Hylon VII starch did not inhibit complex formation. On the contrary, native lipids appear to increase the complexation yield and thermal stability of the starch-fatty acid ester inclusion complexes, possibly due to the formation of ternary complexes.

From the three fatty acid esters studied, only ascorbyl palmitate was entrapped in starch spherulites. Various structures including round spherulites, various sizes of torus-shape spherulites, non-spherulitic birefringent and non-birefringent particles, “balloon” morphologies, and gel-like material were formed depending on processing conditions. However, only the torus-shape spherulites, and some non-spherulitic birefringent and non-birefringent particles showed ascorbyl palmitate entrapment. The % yield of the precipitate increased with higher % of added Hylon VII, and decreased with higher heating temperature and faster cooling rates. The amount of entrapped ascorbyl palmitate in the starch precipitate seems to be governed by the amount of this compound added during processing.

This study showed that starch can form inclusion complexes with fatty acid esters which may be used for the delivery of certain bioactive molecules. In addition, encapsulation of fatty acid esters in starch spherulites may be a good potential delivery system for water soluble bioactive molecules. However, further research is necessary to gain a better understanding of the type of molecules that can be entrapped in starch spherulites, and the factors affecting spherulitic crystallization and bioactive compound entrapment.
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Chapter 1
INTRODUCTION AND OBJECTIVES

1.1. Introduction

The ability of certain bioactive compounds to provide health benefits make these molecules attractive to consumers (Cohen and others 2008). Because of these apparent health benefits, interest in bioactive compounds in foods is growing (Shimoni 2009) resulting in an increasing demand of more nutritive functional foods. However, most phytonutrients found in fruits and vegetables are bitter, acrid or astringent, which makes the use of these compounds a challenge to functional food designers (Drewnowski and Gomez-Carneros 2000). Many bioactive compounds are also sensitive to thermal and oxidative stress and can be easily degraded during processing or storage, and possibly during their passage through the gastrointestinal tract after consumption. In addition, their solubility in water is often limited and, therefore, a vehicle to increase their dispersability in a hydrophilic food environment is necessary. Encapsulation can provide to these compounds increased stability during processing and storage, and may protect the bioactive components during transit through the gastrointestinal tract while providing controlled release properties. Protecting fragile bioactives during transit through the acidic environment of the stomach may insure their release in the small intestine where they are absorbed into the blood stream (Shimoni 2009). In addition, this type of controlled release capsule may prevent the perception of undesirable sensory attributes that are associated with some bioactive compounds.

Fortification of food products with vitamins is a common practice in the food industry. Thus, methods to improve stability of vitamins and their controlled release are of great interest. The relationship between fruit and vegetable consumption and the reduced rates of cancer and coronary heart disease has been widely recognized. For
example, phytosterols, found in fruits and vegetables, are well known to reduce low density lipoprotein-cholesterol levels, lowering the risk of cardiovascular diseases (Moreau and Hicks 2004).

The use of starch in the pharmaceutical industry has grown in recent years, for example, to produce particles with a biodegradable matrix for subcutaneous or nasal delivery (Eliasson and Wahlgren 2004). Amylose (McConnell and others 2007; Milojevic and others 1996a, 1996b) and high amylose starch (Freire and others 2009b; Podczeck and Freire 2008) have also been studied for colon-specific drug delivery in oral formulations, where the controlled release mechanism is the enzymatic degradation of the starch in the colon (Eliasson and Wahlgren 2004; Freire and others 2009a; McConnell and others 2007; Milojevic and others 1996a, 1996b). Biodegradability, low toxicity, food grade, and availability are some of the benefits of using starch for encapsulation purposes. In addition, its physicochemical properties, including retrogradation (recrystallization), film forming ability, complex formation, and resistance at different degrees to amylase hydrolysis, make starch a promising material for delivery systems.

Amylose and amylopectins are well known to form inclusion complexes with some low molecular weight polar and non-polar substances such as iodine, alcohols, fatty acids, aromas (Rondeau-Mouro and others 2004), DMSO (Godet and others 1995b), salicylic acid and its analogues (Oguchi 1998; Uchino and others 2002), p-aminobenzoic acid (Tozuka and others 2006), ibuprofen, warfarin (Hong and others 1998), and organic dyes (Kim and others 1996). When these guest molecules are present, amylose forms a left-handed single helix stabilized by hydrogen bonds (Conde-Petit and others 2006), with a hydrophilic outside surface and a hydrophobic inside helical channel (Immel and Lichtenthaler 2000). The single helical conformation that amylose adopts when it crystallizes is known as V-type (Conde-Petit and others 2006). Guest molecules are usually included within the single helix, but can also be trapped between helices (Rondeau-Mouro and others 2004).
Even though the formation of inclusion complexes of a wide variety of small molecules and amylose has been known for many years, only recently, have these amylose inclusion complexes been seriously investigated as a delivery system of non-drug bioactive compounds (Cohen and others 2008; Lalush and others 2005; Lesmes and others 2008; Lesmes and others 2009; Zabar and others 2009), including conjugated linoleic acid (Lalush and others 2005; Yang and others 2009) and genistein (Cohen and others 2008). These investigations suggested that inclusion complexes with bioactives can protect these molecules during the passage through the stomach, and be released in the small intestine by the action of enzymes on the amylose complexes (Lalush and others 2005; Yang and others 2009).

The formation of amylose-inclusion complexes depends on the size, shape, and solubility of the guest molecules. If guest molecules are too water soluble, for example ascorbic acid, then they will not effectively partition into the starch matrix. In other cases, if the molecules are too large, for example retinol or phytosterols, then they will not fit within the helical cavity that amylose forms when complexing with the ligand. Given the well known ability of amylose to form inclusion complexes with fatty acids, the use of fatty acid esters of these compounds may be an interesting approach for the formation of inclusion complexes with these bioactive molecules.

Encapsulation of bioactive compounds using a commercial native starch would be of relevance because of the lower cost as compared to prepared amylose (Tapanapunnitikul and others 2008). However, the presence of lipids present in native starch can interfere with starch complexation with certain compounds, for example with iodine (Hizukuri and others 2006). In other cases, the presence of native lipids can favor complex formation, such is the case with low-solubility flavors (Tapanapunnitikul and others 2008).

Previous studies have shown that starch inclusion complexes with fatty acids (Bhosale and Ziegler 2009; Byars and others 2003, 2006; Fanta and others 2002; Fanta
and others 2005; Fanta and others 2006; Peterson and others 2005; Shogren and others 2006) or some flavors (Heinemann and others 2003; Heinemann and others 2005) can induce spherulitic crystallization of starch forming small torus-shape and large spherical/lobed particles. Heinemann and others (2003) reported that these types of spherulites are slowly hydrolyzed at low concentrations of α-amylase, while breakdown of spherulites occurs at high α-amylase concentration, suggesting that these types of spherulites can be slowly degraded in the mouth and completely degraded in the gastrointestinal tract.

Another type of starch spherulite can be formed after heating a starch dispersion to temperatures above 170 °C and quickly cooling it to temperatures below 70 °C (Creek and others 2006). These spherulites have a round/spherical morphology and amylose inclusion complexes are not required for their formation (Creek 2007; Creek and others 2006; Nordmark and Ziegler 2002a, 2002b; Suwanayuen 2009; Ziegler and others 2005; Ziegler and others 2003). Previous research showed via confocal scanning laser microscopy successful matrix encapsulation of fluorescein dye in this type of round spherulites (Ziegler, personal communication, May 14, 2008).

Bhoshale and Ziegler (2009) showed that the round spherulites contained 28% resistant starch, while the torus-shape spherulites contained 39% resistant starch. After amylase hydrolysis, pores and an uneven surface were observed in round spherulites, while torus-shape spherulites were degraded, although there was some evidence of recrystallization.

Many factors can affect the spherulitic crystallization of starch. For example, Ziegler and others (2003) reported that a heating treatment above 170 °C and fast cooling are necessary for the formation of well developed round spherulites. Creek and others (2006) hypothesized that this temperature is necessary to go through a helix → coil transition in order to avoid gel formation during cooling caused by the presence of helical nuclei.
Spherulite formation depends also on the starch source (Ziegler and others 2003). Nordmark and Ziegler (2002) studied the spherulitic crystallization of high amylose maize starch (ae 50 and ae 70 genotypes), common corn starch, waxy maize starch, amylose from ae 70, and amylopectin from waxy maize starch. These authors reported that spherulite formation is favored by linear material (amylose) and that spherulites were practically absent in amylopectin samples (waxy maize). Similarly, Creek and others (2006) reported that amylopectin from potato starch did not form spherulites when heating a 10% dispersion to 180 °C and cooling it to 10 °C at cooling rates varying from 1–250 °C/min. Nordmark and Ziegler (2002a) suggested that the lightly branched intermediate material may be ideal for spherulite formation. However, the effect of various proportions of starch fractions from the same botanical source (amylose, amylopectin, and intermediate material) on spherulite formation and microstructure has not been studied.

Bhoshale and Ziegler (2009) reported that morphology of spherulites and entrapment of guest molecules in these particles depended on the processing conditions. These authors suggested that by choosing proper processing conditions, starch spherulites may be tailored for controlled or targeted release delivery systems. However, further research is necessary to fully understand spherulitic crystallization of starch.

1.2. Hypothesis and objectives

The general hypothesis of the project was that: “Fatty acid esters of ascorbic acid, retinol, or phytosterols can be entrapped in starch spherulites by molecular encapsulation in starch inclusion complexes”.

Given the dependence of processing conditions, such as temperature treatment and molecular structure of the starch, on spherulitic crystallization and guest molecule entrapment, the specific objectives of the present study were to: a) investigate the effect
of amylose, amylopectin, and intermediate material on spherulite formation and its microstructure; b) investigate the formation of amylose and high amylose starch inclusion complexes with ascorbyl palmitate, retinyl palmitate, and phytosterol esters; c) evaluate the ability of spherulites to form in the presence of fatty acid esters and to entrap ascorbyl palmitate, retinyl palmitate, and phytosterol esters, and d) evaluate the effect of processing condition on spherulite formation and fatty acid ester entrapment.

1.3. References


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Chapter 2
LITERATURE REVIEW

2.1. Starch Granule

Starch granules are semi-crystalline structures comprising two principal components: amylose and amylopectin, which are polymers of α-D-glucose molecules. Depending on the botanical source, starch granules can markedly differ in various physical and chemical properties including amylose/amylopectin ratio, amylose and amylopectin molecular structure, degree of crystallinity, crystalline polymorph, size (1 μm to 100 μm), and shape (e.g. spheres, discs, ovals, and polyhedral) (Buléon and others 1998).

2.1.1. Amylose

Starch amylose is a polymer formed of glucose molecules connected by α(1-4) linkages. The mostly linear amylose molecule contain less than 1% branching points as α(1-6) linkages. The molecular mass and degree of polymerization (DP) of amylose depends on the botanical source and varies between $10^5$–$10^6$ g/mol, and 324–4920 DP, respectively (Tester and others 2004). Amylose content in the starch granule also depends on the botanical origin. Amylose content in most common types of cereal starches generally varies between 18% and 33% (Buléon and others 1998). However, some plants have been specifically bred to produce starches with particular amylose content (Jenkins and Donald 1995). For example, waxy starches may contain less than 1% amylose, or high amylose starches can contain as much as 70% amylose (Buléon and others 1998). The involvement of amylose in the crystalline structure of granular starch is minimal, but amylose can recrystallize or self-assemble in various forms including
aggregates, precipitates, gels, and spherulites (Buleón and others 2007; Suwanayuen 2009).

2.1.2. Amylopectin

Amylopectin is a highly branched polymer with a typical molecular mass of $10^7$ to $10^8$ g/mol and average DP of 2 000 000. Amylopectin side chains are formed of glucose units connected by $\alpha(1-4)$ linkages. These chains are connected to each other by $\alpha(1-6)$ linkages at the branching points. Figure 2.1 shows a schematic representation of the amylopectin molecule illustrating the generally accepted designation of chain segments A, B, and C (Zobel 1988). The sole reducing group is located on the C-chain. B-chains can be linked through an $\alpha(1-6)$ bond to one or more A and/or B-chains, while A chains are linked with a single $\alpha(1-6)$ bond to the remainder of the molecule (Zobel 1988).

Figure 2.1. Schematic representation of amylopectin molecule showing the A, B and C chain segment designation. The open circle represents the sole reducing end. Adapted from Parker and Ring (2001).
2.1.3. Starch granule structure

The internal structure of native starch granules is characterized by the presence of concentric rings formed of alternating layers of semi-crystalline and amorphous material (Figure 2.2). These rings are known as “growth rings” and have a thickness of about 120–400 nm (Yamaguchi and others 1979). The structure of the semi-crystalline ring is characterized by regularly alternating crystalline and amorphous lamellae. The crystalline and amorphous lamellae combined together have a thickness of 9–10 nm, which appears to be common in all starch granules regardless of the botanical source (Jenkins and others 1993). The amorphous lamella is composed of the branched regions of amylopectin and amylose. The crystalline lamella consists of double helices of amylopectin side chains regularly organized in clusters. Because of the molecular order and radial organization of polymer chains in the starch granules, granules display birefringence and the so called “Maltese cross” when viewed between crossed polarizers (Buléon and others 1998; Gallant and others 1997).

Figure 2.2. Schematic representation of the different structural levels of the starch granule and the involvement of amylose and amylopectin. Reproduced from Buléon and others (1998).
New evidence based on scanning electron microscopy (SEM), atomic force microscopy (AFM), and transmission electron microscopy (TEM) suggests that the crystalline region is actually composed of alternating crystalline and amorphous lamella of amylopectin, organized as a “blocklet” structure (Figure 2.3) (Gallant and others 1997; Ridout and others 2002). The size of the blocklets range between 20 to 500 nm and depends on the botanical source and the location in the granule. For example, in wheat starch, blocklets in crystalline regions are larger (80–120 nm) than those in the semi-crystalline regions. Potato and amylomaize starch have blocklets in the range of 50 nm to 500 nm (Gallant and others 1997; Gallant and others 1992).

2.2. Crystalline allomorphs of starch

Three crystalline structures called A-, B- and C-type can be found in granular starches. In general, A-type crystallinity is observed in cereal starches, B-type in root, tubers, and high amylose starches, and C-type, a mixture of A- and B-type, is observed in legume, root and some fruit and stem starches (Tester and others 2004). Amylose and amylopectin can recrystallize as A- and B-type structures. In general, A-type crystallization is favored by high recrystallization temperatures and short polymer chains, while B-type is induced by low recrystallization temperatures and long polymer chains (Buleón and others 2007).

A- and B-type structures are formed by double helices with identical helical structures (Imberty and others 1991). The way these double helices are packed together is what differentiates these two polymorphic forms (Tester and others 2004). The more compact packing of double helices in the A-type structure only contains 4 water molecules per unit cell, while the more open packing of the B-type structure contains 36 water molecules per unit cell (Figure 2.3).
Figure 2.3. Overview of the granule structure including the blocklet structure and crystal structures of starch polymers. • between helices in type A and B structures at the bottom right of the figure represents water molecules. Reproduced from Gallant and others (1997).
A fourth type of allomorph, known as V-type, can be formed by inducing the formation of an inclusion complex between starch and a guest molecule, such as iodine, dimethyl sulfoxide (DMSO), alcohols, or fatty acids. Unlike A- and B-type structures, the V-type structure is formed by single helices.

2.3. Starch inclusion complexes

Amylose and amylodextrins are well known to form inclusion complexes with some low molecular weight polar and non-polar substances such as iodine, alcohols, fatty acids, aromas (Rondeau-Mouro and others 2004), DMSO (Godet and others 1995b), salicylic acid and its analogues (Oguchi 1998; Uchino and others 2002), p-aminobenzoic acid (Tozuka and others 2006), ibuprofen, warfarin (Hong and others 1998), and organic dyes (Kim and others 1996). When these guest molecules are present, amylose forms a left-handed single helix stabilized by hydrogen bonds (Conde-Petit and others 2006), with a hydrophilic outside surface and a hydrophobic inside helical channel (Immel and Lichtenthaler 2000). These amylose single helices can arrange forming a crystalline structure known as V-type (Biais and others 2006) and it is typically characterized by X-ray diffraction (Godet and others 1993b). Various techniques have been used to investigate these inclusion complexes, such as X-ray and electron diffraction (Bluhm and Zugenmaier 1981; Brisson and others 1991; Buléon and others 1990; Rappenecker and Zugenmaier 1981; Sarko and Biloski 1980), differential scanning calorimeter (DSC) (Biliaderis and Galloway 1989; Biliaderis and others 1985; Eliasson 1994), and solid state NMR (Gidley and Bociek 2002; Horn and others 1987; Kawada and Marchessault 2004; Le Bail and others 2000; Snape and others 1998).

Usually, ligands are included within the single helix but can also be trapped between amylose helices (Rondeau-Mouro and others 2004). In complexes formed with iodine and DMSO, more than one guest molecule is included within the helix (Bluhm and Zugenmaier 1981; William and Anatole 1974). Although complexing molecules are
necessary for the formation of the single helical structure (Godet and others 1996), these molecules may or may not be present in the obtained precipitate (Godet and others 1995b).

Amylose complexes can be classified based on the number of glucose units per helical turn and on the packing of the single helices as shown in Table 2.1 (Conde-Petit and others 2006). The V$_{6l}$ or V$_{h}$ conformation comprises six glucose units per helical turn and has a rise per monomer between 1.32 Å and 1.36 Å (Le Bail and others 1995) with a repeating distance between adjacent turns of the same helix (fiber repeat) of 8.05 Å (Brisson and others 1991; Rappenecker and Zugenmaier 1981). Water molecules are located within and between helices forming a network of hydrogen bonds (Brisson and others 1991; Rappenecker and Zugenmaier 1981) which stabilizes the helices (Eliasson and Wahlgren 2004). Specific conditions of hydration and temperature can give rise to transitions between amylose polymorphs (Le Bail and others 1995). At water activities lower than 0.6, V$_{h}$ complexes can convert to a V$_{a}$ complex (anhydrous form) (Hinkle and Zobel 1968; Murphy and others 1975; Rappenecker and Zugenmaier 1981). This transition is reversible by hydration (Zaslow and Miller 1961). The transition between V$_{a}$ to V$_{h}$ has been suggested to be caused by an increase in the distance between helices (Rappenecker and Zugenmaier 1981; Zaslow and others 1974) accommodating water molecules (Le Bail and others 1995). V$_{h}$ polymorph can also transition into A, B, or mixtures of A and B polymorphs when rehydrating at higher water activities (>84% RH). The conformation that V$_{h}$ will adopt at these higher water activities depends on the chain length of amylose. V$_{h}$ complexes made with short chain amylose (DP 20) reorganize into A-type polymorph, while those formed with longer chains (>DP 80) reorganize into the B-type form (Le Bail and others 1995). Reorganization into A- and B-type forms are permanent transformations unless the sample is dissolved again (Kawada and Marchessault 2004).
Table 2.1. Classification of V-type amylose complexes (adapted from Conde-Petit and others 2006).

<table>
<thead>
<tr>
<th>Type</th>
<th>General properties</th>
<th>Example of ligands</th>
<th>Reference</th>
</tr>
</thead>
</table>
| $V_{6I}$ or $V_{h}$ | Six glucose units per helical turn  
Except for ethanol, guest molecules are located within helical cavity.  
Increased thermostability of complexes as the chain length of the ligand increases | Fatty acids                  | (Godet and others 1995a; Godet and others 1996; Godet and others 1993a) |
|              |                                                                                    | Ethanol                     | (Brisson and others 1991)                                                 |
|              |                                                                                    | Decanal                     | (Le Bail and others 2005; Nuessli and others 1997)                        |
|              |                                                                                    | Lactones                    | (Heinemann and others 2001)                                               |
|              |                                                                                    | Limonene                    | (Tapanapunnitikul and others 2008)                                       |
|              |                                                                                    | Cymene                      | (Tapanapunnitikul and others 2008)                                       |
| $V_{6II}$ or $V_{butanol}$ | Six glucose units per helical turn  
Guest molecules located within helical cavity | Butanol                     | (Helbert and Chanzy 1994; Le Bail and others 2005; Nuessli and others 1997) |
| $V_{7}$, $V_{6III}$ or $V_{isopropanol}$ | Guest molecule position is not clear.  
Earlier studies proposed the location of the guest molecule within the helix with seven glucose units per turn, which could not be confirmed.  
Recent studies suggested six glucose units per helical turn with the location of guest molecules between helices. | Isopropanol                  | (Buléon and others 1990; Nuessli and others 2003) |
|              |                                                                                    | Menthone                    | (Le Bail and others 2005; Nuessli and others 2003; Rondeau-Mouro and others 2004; Tapanapunnitikul and others 2008) |
|              |                                                                                    | Fenchone                    | (Nuessli and others 2003)                                                 |
|              |                                                                                    | Geraniol                    | (Tapanapunnitikul and others 2008)                                       |
|              |                                                                                    | Thymol                      |                                                                            |
|              |                                                                                    | Linalool                    | (Arvisenet and others 2002; Rondeau-Mouro and others 2004)                |
|              |                                                                                    | Ethyl hexanoate             | (Arvisenet and others 2002)                                               |
|              |                                                                                    | Genistein                   | (Cohen and others 2008)                                                   |
|              |                                                                                    | Salicylic acid at low concentration |                                      | (Oguchi 1998)               |
| $V_{8}$      | Eight glucose units per helical turn  
Guest molecule is included within helical cavity and between helices.  
Largest helix diameter for amylose complexes | $\alpha$-Naphtol            | (Le Bail and others 2005)                                                 |
|              |                                                                                    | Salicylic acid at high concentration |                                      | (Oguchi 1998)               |

Nuessli and others (1997) reported that various flavor molecules with similar size and shape significantly varied in thermostability and crystallinity of their complexes with starch. The authors suggested that other properties of the guest molecules, such as polarity or solubility, may be affecting crystal structure and thermal stability of their complexes. For example, higher yield and flavor entrapment was observed for flavors
with higher water solubility as compared to that of flavors with lower water solubility (Tapanapunnitikul and others 2008).

Inclusion complexes can crystallize with a lamellar organization. The lamella folded chain length has been reported to be about 10 nm for amylose complexes formed with alcohols (Jane and Robyt 1984). Similarly, 10 nm helical segments has been also reported for amylose-monoglyceride complexes formed at 60 °C, while those formed at 90 °C had a helical chain segment of 14.5 nm (Galloway and others 1989). These studies assumed that the size of amylodextrin fragments resistant to α-amylase corresponded to the thickness of the crystals. (Godet and others 1996). However, determination of crystal thickness of amylose-fatty acid complexes by transmission electron microscopy and image processing showed that crystal thickness increased with fatty acid chain length and amylose DP and was always less than 4.6 nm (length of two palmitic acid molecules) (Godet and others 1996).

2.3.1. Starch-lipid complex

Amylose-lipid complexes have been widely studied in the past years. Various techniques have been used to elucidate the structure of these inclusion complexes, such as X-ray diffraction, electron diffraction (Brisson and others 1991), molecular modeling (Godet and others 1993b), Raman spectroscopy (Carlson and others 1979), and 13C-cross polarization/magic-angle spinning nuclear magnetic resonance spectroscopy (CP/MAS NMR) (Morrison and others 1993). The model that is currently accepted is that the central cavity of the amylose helix is occupied by the aliphatic chain of the lipid. The head group is not included within the helix because the diameter of the internal cavity is not large enough to accommodate bulky groups (Godet and others 1995b). Based on molecular modeling, Godet and others (1993b) proposed two stable conformations in which the carboxylic group is located close to the entrance of the helical cavity. Steric and electrostatic repulsions prevent the polar group to enter the helical cavity. Lebail and
others (2000) based on their research of amylose complexes with palmitic and lauric acid studied with \(^{13}\)C CP/MAS NMR, deuterium NMR, X-ray powder diffraction, and DSC, proposed a partially disordered model of the amylose-lipid complex structure. The model comprises the fatty acid partly inside and partly outside the helical cavity, depending on the crystallization conditions and the requirement of having the carboxylic group outside the helix (Le Bail and others 2000). The \emph{all-trans} configuration of the saturated fatty acid inside the helix is the most thermodynamically favored conformation (Godet and others 1993b), but based on CP/MAS NMR studies, Le Bail and others (2000) suggested that part of the palmitic acid might be in a \emph{gauche-trans} configuration and part of the alkyl chain is outside the helix. These authors explained that both forms may exist due to the small differences between the energy of the two configurations (Le Bail and others 2000).

The presence of fatty acids between amylose helices in the amorphous region is unlikely (Godet and others 1995b). Molecular modeling based on two structural models of the \(V_{h}\)-type crystal proposed by Brisson and others (1991) and Rappenecker and Zugenmaier (1981) showed that the spaces between amylose helices are too small to fit the alkyl chain of fatty acid molecules. The presence of fatty acid molecules between helices would result in distortion of the crystalline unit cell, and thus, a shift of corresponding X-ray diffraction peaks (Godet and others 1995b), similar to the arrangement proposed for isopropanol complexes (Buléon and others 1990; Godet and others 1995b). Smaller molecules, such as water or short alcohols, are more compatible with these voids between helices (Godet and others 1995b).

Amylose forms a single left-handed helix around the monoacyl chain. The helix is formed by 2–3 helical turns with 6 glucose units per turn and approximately 8.1 Å repeating distance between adjacent helical turns of the same helix. The length of the helix is likely to be limited by the lipid structure (Eliasson and Wahlgren 2004). Molecular modeling based on X-ray diffraction data for iodine, suggested that the overall diameter of the helix is approximately 13.5 Å, and the width of the interior of the helix is
about 5.4 Å (Immel and Lichtenthaler 2000). The general agreement is that saturation of the complex is reached at around 10% lipid (w/w amylose) (Le Bail and others 2000; Raphaelides and Karkalas 1988), resulting in free, uncomplexed lipids if higher proportions are added.

Amylose-lipid complexes have been reported to have various polymorphic forms which depend on the ligand and temperature of complex formation (Eliasson 1994; Gelders and others 2005; Kowblansky 1985). These polymorphic forms are described as low-melting form, also called type I or form I, and high-melting form, also called type II or form II (Biliaderis 1992; Biliaderis and Galloway 1989; Eliasson 1994). Type I inclusion complexes are formed at low crystallization temperature (Biliaderis and Seneviratne 1990), have little crystallographic register (amorphous X-ray diffraction pattern), and are assumed to be formed by helical segments randomly distributed in wet samples (Biliaderis 1992; Biliaderis and Galloway 1989). However, a diffused V-type pattern can be observed after freeze-drying and rehydrating type I complexes (Seneviratne and Biliaderis 1991). On the other hand, type II complexes are polycrystalline aggregates that display the characteristic V-type X-ray diffraction pattern, have birefringent properties, and a more compact organization than type I complexes (Biliaderis 1992).

Type II complexes are thought to have the amylose complexes organized in a lamellar structure, in which the chain axes are perpendicular to the surface of the lamella (Biliaderis 1992; Biliaderis and Galloway 1989). Two forms of type II complexes, IIa and IIb, have also been differentiated based on their melting temperatures. Type I inclusion complexes can convert to type II complexes only if partial melting of their structure occurs (Biliaderis and Galloway 1989; Biliaderis and Seneviratne 1990; Tufvesson and Eliasson 2000). Even though type I and II complexes differ in their melting temperature and degree of crystallinity, the enthalpy of the endothermic transition remains constant indicating that the overall energy of the transitions is mainly due to the conformational dissociation of the helices, with little effect from the interchain
interactions (Biliaderis 1992; Biliaderis and Seneviratne 1990). These two type of complexes melt cooperatively (Eliasson and Wahlgren 2004).

The melting temperature of these complexes depends on the structure of the ligand. The longer the monoacyl chain, the higher the melting temperature. On the contrary, lower melting temperature is observed as the degree of unsaturation increases. Diacyl lipids, such as lecithin, can also form inclusion complexes with amylose (Kowblansky 1985), but complex formation with triglycerides has not been observed (Eliasson 1994).

Thermal behavior of inclusion complexes are also affected by the processing parameters used during thermal analysis. For example, if water content is lowered below a certain limit, the melting temperature of the complexes increases. This effect has been attributed to the water’s plasticizing effect on the glass transition temperature and thus on the melting temperature of the crystals (Biliaderis and others 1986a). Water content not only affects the melting temperature, but at lower water contents, the appearance of double peaks with an exothermic transition between them has been observed. This thermal behavior has been interpreted as the melting and recrystallization of the complexes (Biliaderis and others 1986a; Biliaderis and others 1986b; Biliaderis and others 1985) into a thermodynamically more stable conformation (lower free energy) (Biliaderis and Seneviratne 1990). At lower moisture content, melting is less cooperative and occurs at higher temperatures, increasing the probability of a secondary crystallization. In addition, at low water content less perfected structures partially melt in a temperature region where recrystallization occurs at a high rate. On the other hand, single endothermic transitions are observed at water contents higher than 80% (Biliaderis and others 1986a).

2.3.1.1. Formation of starch-lipid complexes

Starch-lipid complexes are formed through a number of processes:
The transition from coil to helix: hydrogen bond formation between glucose units and water exclusion from inside the helix

The hydrophobic interactions between the hydrocarbon chain of the lipid and the internal cavity of the helix

The formation of type I or type II structures

In order to form the complex, the lipid needs to be free in solution. Hence, the amount of complex formed will depend on factors that influence solubility of the lipid in the reaction medium. For example, at high pH, fatty acids are fully charged and their tendency to form complexes is larger (Eliasson and Wahlgren 2004).

Factors affecting the formation of amylose-lipid inclusion complexes

Preparation technique and temperature treatment

Various methods to achieve co-crystallization of amylose with guest molecules to form inclusion complexes have been used: 1) Heating an aqueous dispersion of amylose and lipid in sealed DSC pans followed by crystallization on cooling; 2) solubilizing amylose in alkali solution followed by neutralization (Raphaelides and Karkalas 1988); and 3) solubilizing amylose and lipid in hot DMSO aqueous solution followed by slow cooling after water addition (Raphaelides and Karkalas 1988). The method used can affect the efficiency of complexation. For example, Raphaelides and Karkalas (1988) reported that a fraction of the fatty acids remained uncomplexed when using the DMSO complexation method, while uncomplexed fatty acids where not detected using the alkali solution method. The preparation technique can also affect the thermal properties of the amylose-fatty acid complex polymorphs (Raphaelides and Karkalas 1988).

The temperature treatment affects the type of complex formed (Karkalas and others 1995; Tufvesson and Eliasson 2000; Tufvesson and others 2003a, 2003b). The complexation temperature generally used to form type I and type II complexes is 60 °C.
and 90 °C, respectively (Gelders and others 2005). For the formation of type II complexes with longer and unsaturated monoglycerides, a prolonged heat treatment (24 h at 100 °C) is necessary, while with short monoglycerides, type II complexes can be formed by heating from 15 °C to 144 °C at 10 °C/min. (Tufvesson and others 2003a).

Starch properties

Starch-inclusion complex formation is affected by the botanical origin of the starch. Starches from different sources can affect the heat stability and how easily the complexes are formed under non-ideal conditions (Eliasson 1994), likely due to the differences in the proportion of amylose and amylopectin and their molecular structure. The ability of amylose to form inclusion complexes with certain lipids is well known. On the other hand, evidence on the ability of the branched amylopectin molecule to form lipid inclusion complexes is scarce (See section 2.3.1.2). If amylopectin-lipid complexation occurs, only one lipid molecule will probably bind to an individual amylopectin chain (Eliasson and Wahlgren 2004).

Complex formation is influenced by the chain length of the starch molecule. Godet and others (1995a) studied the effect of various amylose DP, between 20 and 900 glucose units, on complex formation with fatty acids. These authors concluded that longer chains produce larger amounts of complexes with higher dissociation temperature. They also reported that precipitates were not obtained with DP 20 amylose.

Lipid structure

The formation of inclusion complexes are greatly affected by the molecular structure of the lipid, including aliphatic chain length, head group, and degree of unsaturation.
Fatty acids with very short chain length, such as propionic (C3) and butyric acid (C4) cannot form inclusion complexes with amylose (Tufvesson and others 2003a). Longer aliphatic chains result in higher complexation with amylose (moles of lipids bound per mole of amylose) (Hahn and Hood 1987). As the alkyl chain increases, the thermal stability (i.e. dissolution temperature) (Eliasson and Krog 1985; Godet and others 1996; Kowblansky 1985; Tufvesson and others 2003b) and crystal thickness of the produced complexes increases (Godet and others 1996). However, the dissociation enthalpy has been reported to be practically independent of the chain length (Raphaelides and Karkalas 1988).

The polar head group of the lipid affects the thermal stability of the inclusion complex. In general, amylose-monoglyceride inclusion complexes have a lower dissociation temperature than their fatty acid counterparts (Tufvesson and others 2003b).

The ability of amylose to form inclusion complexes with unsaturated fatty acids with various degrees of unsaturation has been reported (Eliasson and Krog 1985; Karkalas and others 1995; Karkalas and Raphaelides 1986; Snape and others 1998; Zabar and others 2009). Thermal stability of amylose-lipid complexes decreased with increasing unsaturation (Eliasson and Krog 1985; Tufvesson and others 2003b). In general, unsaturated fatty acids are less effective in forming inclusion complexes as compared to their saturated counterparts (Hahn and Hood 1987; Karkalas and Raphaelides 1986; Zabar and others 2009). For each double bond in the fatty acid guest molecules, the dissociation temperature significantly decreased (Karkalas and others 1995).

Zabar and others (2009) studied the effect of the molecular structure of the fatty acid on the inclusion complex formation and nanostructure. In this study, the authors evaluated various fatty acids with 18 carbons varying the position and number of double bonds. Increasing degree of unsaturation of long chain fatty acids resulted in the formation of ill-defined crystals compared to the crystalline structure of saturated fatty
acids. Based on small angle X-ray scattering studies, the structure of amylose-fatty acid complexes is a region of amorphous material in which crystalline regions, assumed to be formed by amylose chain folded lamellae, are embedded (Zabar and others 2009). The percentage of crystallinity of amylose-saturated fatty acid complexes prepared in DMSO at 90 °C has been reported to be 45–59% (Godet and others 1995a). The amorphous regions that separate the crystalline lamellae are composed of regions of imperfections, chain ends, and possibly residues of fatty acids (Zabar and others 2009). Increasing chain flexibility (i.e. higher degree of unsaturation) resulted in a decrease in the order within the lamellae: lower crystalline fraction, smaller average crystalline lamellar thickness (Zabar and others 2009), and bigger and more dispersed particle population (Lesmes and others 2009).

2.3.1.2. Amylopectin-lipid complex

Several studies have shown evidence that amylopectin may interact with lipids or surfactants (Eliasson and Ljunger 1988; Evans 1986; Gudmundsson and Eliasson 1990; Hahn and Hood 1987; Huang and White 1993; Kim and others 1992; Nakazawa and Wang 2004; Villwock and others 1999). Villwock and others (1999) showed as evidence of the existence of amylopectin-lipid complexes the reduction of the enthalpy of gelatinization of waxy maize in the presence of lipid. These authors explained that the formation of starch-lipid complexes occurring simultaneously during starch gelatinization is an exothermic transition, which is believed to be the cause of the reduction of the enthalpy of gelatinization. However, when samples were rescanned in the DSC, an endothermic transition characteristic of complexes could not be observed (Villwock and others 1999).

Gudmundsson and Eliasson (1990) reported the formation of complexes between potato amylopectin and emulsifiers with an endothermic transition above 100 °C, which is the usual transition temperature for amylose-lipid complex. Interaction of monoglycerides with waxy corn starch (Huang and White 1993) and potato amylopectin
(Batres and White 1986) have also been previously reported. This interaction was assumed to be an amylopectin-lipid complex based on the lower iodine affinity compared to the control. Amylopectin chains that are complexed with the lipid will not be available to bind iodine, reducing the iodine capacity of the sample (Huang and White 1993).

Even though previous studies support the interaction between amylopectin and lipids, the specific nature of this interaction is little known (Villwock and others 1999). For example, no conclusions about the nature of the interaction between monoglycerides and potato amylopectin was possible based on infrared and NMR spectra (Batres and White 1986). Single helices of amylopectin-lipid inclusion complexes are probably very similar to that of amylose, but differed in their organization and supramolecular structure (Eliasson and Wahlgren 2004).

2.3.1.3. Enzyme susceptibility of starch-lipid complexes

In-vitro studies have shown that amylose-lipid complexes are highly resistant to $\alpha$-amylase hydrolysis as compared to free amylose. This difference in enzyme susceptibility was suggested to be possibly due to the decrease in solubility of amylose and the conformational hindrance to enzymatic attack of the V-type structure (Holm and others 1983). However, complexes are completely hydrolyzed after 3 h in excess enzyme conditions (3000 units/g amylose).

The different type of inclusion complexes (i.e. type I and type IIa and IIb) differ in their susceptibility to the action of $\alpha$-amylase. For example, amylose inclusion complexes with glycerol monostearate subjected to hydrolysis using Bacillus subtilis and pancreatic $\alpha$-amylase showed that type I complex had higher rate and extent of complex degradation, followed by type IIa and then type IIb complexes. The difference in enzyme susceptibility was attributed to the degree of organization and perfection of the crystalline domains formed by the aggregated chains (Seneviratne and Biliaderis 1991).
Enzyme susceptibility is also affected by the structure of the ligand. Amylose complexes with lipids with shorter chains are less resistant to enzymatic (Eliasson and Krog 1985; Gelders and others 2005) and acid hydrolysis (Gelders and others 2005). Higher degree of unsaturation of the lipid reduces the resistance of the complex to enzymatic hydrolysis (bacterial α-amylase and amyloglucosidase) compared to complexes with long saturated monoglycerides (Eliasson and Krog 1985). Double bonds of unsaturated fatty acids may cause disturbance in the structure of inclusion complexes reducing their resistance to enzymatic hydrolysis (Holm and others 1983). Increasing the chain length of amylose or using higher complexation temperatures when forming the inclusion complexes, will also result in complexes with higher resistance to enzymatic and acid hydrolysis (Gelders and others 2005).

Even though in-vitro studies show lower enzymatic susceptibility of lipid-inclusion complexes, in-vivo studies showed that amylose-lysolecithin inclusion complexes are not present in the rat gastrointestinal tract after 2 h. However, levels of glucose and insulin levels in plasma after ingestion of free amylose were significantly higher than those following ingestion of amylose complexes. These results suggested that the degradation of amylose complexes is slower in-vitro and in-vivo compared to free solubilized amylose (Holm and others 1983).

Amylose-fatty acid complexes have also been shown to have higher resistance to the action of glucoamylase as compare to that of granular starch. This resistance decreased with degree of unsaturation of the fatty acids (Kitahara and others 1996).

2.4. Starch spherulites

Spherulites have been described as approximately radially-symmetric semi-crystalline structures. When these structures are viewed between crossed polarizers, they exhibit a characteristic “Maltese cross” extinction pattern (Creek and others 2006; Ziegler...
and others 2003). During spherulite formation, long crystals (lamellae) grow from a central nuclei in an approximately radial arrangement (Phillips 1994). Spherulite formation has been observed in various biomolecules, such as collagen (Martin and others 2003), cellulose (Kobayashi and others 2000), β lactoglobulin (Bromley and others 2005), silk fibroin (Tanaka and others 2001), chitin (Murray and Neville 1997, 1998; Sakamoto and others 2000) and chitosan (Murray and Neville 1998), short chain amylose (DP = 15–20) (Helbert and others 1993; Leloup and others 1991; Planchot and others 1997; Ring and others 1987; Whittam and others 1990; Williamson and others 1992), potato amylose (Creek and others 2007), common corn amylose (Creek and others 2007; Creek and others 2006) and starches from various botanical sources (Fanta and others 2002; Fanta and others 2005; Fanta and others 2006; Nordmark and Ziegler 2002a, 2002b; Peterson and others 2005; Shogren and others 2006; Ziegler and others 2005; Ziegler and others 2003).

The formation of two types of spherulites from starches has been reported. The first type has a spherical morphology and amylose inclusion complexes are not required for spherulite formation (Creek 2007; Creek and others 2006; Nordmark and Ziegler 2002a, 2002b; Suwanayuen 2009; Ziegler and others 2005; Ziegler and others 2003). In the second type, spherulitic crystallization is induced by amylose inclusion complexes, and small torus-shape and large spherical/lobed particles are mainly formed (Bhosale and Ziegler 2009; Byars and others 2003, 2006; Fanta and others 2002; Fanta and others 2005; Fanta and others 2006; Peterson and others 2005; Shogren and others 2006).

2.4.1. Spherulites that do not require the presence of amylose inclusion complexes for their formation

Two methods to produce starch spherulites that do not require the presence of amylose inclusion complexes for their formation have been reported. The first method comprises the formation of spherulites from short chain amylose, which is dispersed in
water and heated to 120 °C followed by crystallization on cooling (Helbert and others 1993; Leloup and others 1991; Planchot and others 1997; Ring and others 1987; Whittam and others 1990; Williamson and others 1992). The second method includes heating a starch dispersion to above 170 °C and cooling the dispersion at a relatively fast rate (Ziegler and others 2003).

Spherulites made from short chain amylose (DP = 15–22) with A- and B-type diffraction patterns have been produced. For the formation of B-type spherulites, an aqueous solution of short chain amylose (5–20%) (Ring and others 1987; Whittam and others 1990) is heated to 120 °C, held at this temperature for 30 min, cooled to 80 °C, filtered, and finally cooled to 5 °C at a rate of 5 °C/h (Whittam and others 1990). For the formation of A-type spherulites, the heated starch dispersion is cooled to 80 °C, then warm ethanol is added before cooling to 5 °C (Whittam and others 1990). A-type spherulites are formed by a series of elongated crystals that grow from the center giving the spherulite an inner radial organization. These elongated crystals have spearhead-shape end, which provide the surface of the spherulite with an array of sharp ridges and valleys (Helbert and others 1993). The melting temperature of these spherulites (A- and B-type) decreases at higher water contents. Above 40% water content, A-type spherulites have a melting temperature of approximately 20 °C higher than B-type spherulites (Whittam and others 1990).

Spherulites can also be formed when a 10–20% (w/w) starch or amylose dispersion is heated above 170 °C followed by fast cooling (Ziegler and others 2003). Ziegler and others (2003) reported that a heating treatment above 170 °C and fast cooling are necessary for the formation of well developed spherulites. Creek and others (2006) hypothesized that this temperature is necessary to go through a helix → coil transition in order to avoid gel formation during cooling caused by the presence of helical nuclei. These authors proposed that during heating, when the dispersion reaches around 70 °C, double helical crystallites “dissolve” and by 130 °C, thermal dissociation of fixed network entanglements occurs to form a liquid crystalline phase. Then, between 160 °C
and 180 °C, the solution becomes isotropic, which is necessary for spherulite formation on cooling (Creek and others 2006). During cooling, first, phase separation should be induced, resulting in a polymer-poor phase, and a polymer-rich phase in which spherulitic crystallization occurs. At slow cooling rates, crystallization can occur before phase separation, which may result in network formation preventing demixing (Ziegler and others 2005).

Some structural features reported for spherulites made from high amylose corn starch are radially-oriented crystalline lamellae, and the presence of a central cavity and small holes away from the center (Nordmark and Ziegler 2002b). The estimated degree of crystallinity of starch spherulites ranges from about 20% (Creek and others 2006) to 75% (Creek 2007). Spherulites with B-type crystallinity have mainly been observed when making spherulites using this method (i.e. heating above 170 °C followed by fast cooling) (Creek and others 2006; Ziegler and others 2005). The formation of spherulites without a V-type diffraction pattern suggests that complexes between amylose and native lipids are not responsible for spherulitic crystallization (Ziegler and others 2005). This was confirmed when Creek and others (2007) observed that when L-α-lysophosphatidylcholine (LPC) was added to potato or common corn amylose dispersion, amylose-LPC complexation occurred, but spherulites were not formed. Nordmark and Ziegler (2002a) suggested that amylose lipid complexes are not necessary for spherulite formation, but it may be possible to have these complexes within the spherulite.

2.4.2. Factors affecting spherulite formation

**Starch source**

The botanical origin of the starch affects spherulite formation. In general, A-type starches do not form spherulites easily as compared to B- or C-type starches (Ziegler and others 2003):
- Well developed spherulites can be obtained from mung bean, potato and high amylose maize starches.
- Less numerous and less developed, coarse spherulites are formed from common corn starch.
- Very poorly developed or few spherulites are formed from wheat, tapioca, and arrow root starches.
- Spherulites were not observed in oat, rice, and sorghum starch samples.
- Clear conclusions about spherulite formation from amaranth and yellow pea starches could not be made.

The greater degree of polymerization of amylose from mung bean and potato starch was suggested to be related to their ability to form spherulites as compared to maize and rice starches (Ziegler and others 2003).

**Effect of starch fraction**

Nordmark and Ziegler (2002a) studied spherulitic crystallization from maize starch and its fractions. These authors used amylose from high amylose maize starch (\(ae\) 70) and amylopectin from waxy maize for this study. In that study, the authors showed that linear material (i.e. amylose) favors the formation of spherulites, which have a higher melting temperature and melting enthalpy than those formed from \(ae\) 70 starch. On the other hand, spherulites were practically absent in amylopectin samples (waxy maize) (Nordmark and Ziegler 2002a). Similarly, Creek and others (2006) reported that amylopectin from potato starch did not form spherulites when heating a 10% dispersion to 180 °C and cooling it to 10 °C at cooling rates varying from 1–250 °C/min.

Nordmark and Ziegler (2002a) also suggested that the lightly branched intermediate material may be ideal for spherulite formation. However, the effect of this intermediate material on spherulite formation has not been studied.
**Effect of starch concentration**

The concentration of the starch dispersion affects spherulite formation. Increasing starch concentration results in an increase in the formation of interconnected gel-like material, and a decrease in the formation of spherulites (Creek 2007). Low starch concentrations (5% w/w) may form spherulites depending on the starch source. For example, spherulites with a broad particle size distribution were observed when made from aqueous dispersions of 5% (w/w) high amylose corn starch (Nordmark and Ziegler 2002a), while spherulitic crystallization could not be attained when potato starch was used at this concentration (Ziegler and others 2003). The minimum material concentration to achieve spherulite formation depends on the chain length of the material. Creek (2007) observed spherulite formation when using 5% corn amylose with a chain length between 70 to 930 DP, however a minimum concentration of 25% was necessary when 35 DP corn amylose was used. Spherulites are most easily formed from starch dispersions with concentrations from 10 to 20% (w/w) (Ziegler and others 2003), and the concentration above which no spherulites can form ranges from 35–60% and depends on the DP of the material (Creek 2007).

Thermal properties of spherulites are affected by starch concentration. In general, an increase in starch concentration from 5% to 10% (w/w) does not affect melting temperature of spherulites (Creek 2007), but an increase in concentration from 10% to 20% (w/w) raises DSC melting peak temperature by an average of about 10 °C (Creek 2007; Creek and others 2006). Starch concentration can also affect the spherulite crystalline allomorph. Creek (2007) suggested that increasing the concentration of amylose (DP 70–930) from 20% to about 50–60% can result in the transition from B-type to C-type crystallinity.
Starch concentration also affects the average spherulite size (Creek 2007) and shape (Nordmark and Ziegler 2002a). Creek (2007) observed that the average diameter of corn amylose spherulites increased at higher amylose concentration, independently of the DP of the material. However, above 30% starch, the shape of the spherulites gets distorted because spherulites impinge on one another (Nordmark and Ziegler 2002a).

**Effect of heating temperature**

The minimum heating temperature necessary to form starch spherulites has been reported to be 170 °C (Ziegler and others 2003). Recently, mung bean starch spherulites could be observed when heating a 10% starch dispersion to 166 °C using a DSC (Bhosale, personal communication, July 27, 2008) but not when heating below 164 °C (Ziegler, personal communication, January 11, 2010). Ziegler and others (2003) discussed possible ways the maximum temperature during heating may affect spherulite formation:

- The maximum temperature has to be high enough to assure complete dissolution of the native granule. Vesterinen and others (2001) reported this dissolution to happen above 150 °C.
- High temperatures results in thermal degradation of starch, reducing the amount of branch material and hence increasing the amount of short-chain linear molecules.
- Heating temperature can also increase chain flexibility, which may result in chain folding lamella upon fast cooling.

The idea that high temperature causes some starch degradation was confirmed by Nordmark and Ziegler (2002b) who showed that only traces of amylopectin and intermediate material is present in spherulites made from high amylose starch produced with 3 heating cycles to 180 °C. These authors suggested that amylopectin was thermally degraded during processing. The same authors also suggested that the products from this degradation were the reason for the presence of some spherulites when rapidly cooling a waxy maize solution heated to 180 °C (Nordmark and Ziegler 2002a). However, the
extent of this degradation is not that extreme to produce short chain amylodextrins (DP < 25) like those previously observed to form spherulites (Helbert and others 1993; Ring and others 1987). Creek and others (2006) calculated the degree of polymerization of amylose chains after heating to 180 °C and cooling to 10 °C at 10 °C/min to be 635 (initial DP = 925).

**Effect of final temperature**

Spherulite formation depends on the final temperature on cooling. For starch spherulites, a temperature below 60–70 °C is necessary for spherulitic crystallization (Creek and others 2006; Ziegler and others 2005). Creek and others (2006) reported that spherulites were not formed when cooling the heated starch dispersion above 70 °C, instead, a morphology described as “salt-and-pepper like” and “large feather like” crystalline structures were observed. At 70 °C, a transitional morphology of poorly developed spherulites was obtained. At 60 °C and below, well developed spherulites were formed increasing their size as the holding period at the final temperature was increased (Creek and others 2006). Similarly, Ziegler and others (2005) observed that the final cooling temperature affected the formation of mung bean starch spherulites: crystalline structures were not formed above 85 °C, large birefringent sheets were formed above 65 °C, and spherulites were formed below 65 °C, being more numerous and better defined at lower final temperatures.

**Effect of cooling rate**

The effect of cooling rate on spherulite formation is not well understood. On one hand, it appears that very slow cooling rates are necessary for the formation of spherulites with inclusion complexes. On the other hand, spherulites that do not required the presence of inclusion complexes for their formation are not formed at very slow cooling rates (below 1°C/min) as shown in Table 2.2.
Table 2.2. Effect of cooling rate on the development of spherulites that do not required the presence of inclusion complexes for their formation

<table>
<thead>
<tr>
<th>Cooling rate</th>
<th>Material</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1–1 °C/min</td>
<td>Hylon VII (Nordmark and Ziegler 2002a)</td>
<td>- Spherulites of varying sizes were rarely observed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Formation of birefringent rod-shaped particles or irregular fuzzy clusters, without displaying the characteristic Maltese cross</td>
</tr>
<tr>
<td>1–2.5 °C/min</td>
<td>Mung bean starch (Ziegler and others 2005)</td>
<td>- Spherulite formation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Large increase in volume of non spherulitic “salt and pepper” like material with weak birefringence</td>
</tr>
<tr>
<td>5–10 °C/min</td>
<td>Mung bean starch (Ziegler and others 2005)</td>
<td>- Spherulite formation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Branched crystalline morphologies</td>
</tr>
<tr>
<td>Extremely fast cooling rate by direct immersion of sample pans at 180 °C in liquid nitrogen</td>
<td>Mung bean starch (Ziegler and others 2005)</td>
<td>- Very few spherulites</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Large amount of amorphous material</td>
</tr>
<tr>
<td>Cool from 180 °C to 130 °C at 250 °C/min followed by cooling to 10 °C at various cooling rates</td>
<td>Amylose from normal corn starch (Creek and others 2006)</td>
<td>- Substantially more spherulitic material</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Much less gel-like material</td>
</tr>
</tbody>
</table>

In general, spherulitic crystallization is prevented by extremely fast or slow cooling rates. The limited time for diffusion and reorganization of components at extremely fast cooling rates, and a thermodynamically favored near-equilibrium rearrangement at extremely slow cooling rates do not allow spherulites to form (Nordmark and Ziegler 2002a). Faster cooling rates resulted in more spherulites and a decrease in the amount of non-spherulitic material which exhibited little birefringence and did not showed any additional thermal event in DSC analysis (Creek and others 2006).

Cooling rate also affects the size of the spherulites. However this effect depends on the starch source. Size of spherulites made from common maize amylose was not affected by cooling rate. However, spherulite size increased at faster cooling rates in
spherulites made from normal maize starch (Creek and others 2006) and mung bean starch (Ziegler and others 2005). At cooling rates above 50 °C/min, the diameter of mung bean starch spherulites appeared to be constant (Ziegler and others 2005).

The effect of the cooling rate on thermal properties of spherulites seems to depend on the starch source. For example, peak dissolution temperature of mung bean starch spherulites increases with faster cooling rates, while the peak temperature and melting enthalpy of normal corn amylose spherulites were not significantly affected by cooling rate (Creek and others 2006).

2.4.3. Spherulites formed from amylose inclusion complexes

The formation of spherulites with two types of crystalline morphologies has been reported when common corn and high amylose corn starch dispersions were heated to 140 °C by steam jet cooking, followed by slow cooling (Fanta and others 2002; Fanta and others 2005; Peterson and others 2005; Shogren and others 2006). These structures also formed when high amylose, rice or wheat starch, were processed under the same conditions. However, they were rarely observed when defatted high amylose starch was used, and were not formed with defatted normal corn, waxy maize, or potato starch (Fanta and others 2002). Because these spherulites were not observed when starch with low lipid content (i.e. waxy maize, potato starch) and defatted starches were used, crystallization of inclusion complexes from amylose and native lipid present in cereal starch granules was suggested as the cause of the formations of these spherulites (Fanta and others 2002). In general, these type of spherulites form when a starch solution is heated in the presence of guest molecules to temperatures below 150 °C, cooled to 90 °C (rate may vary), then slowly (~2.5 °C/h) cooled to temperatures below 40 °C (Bhosale and Ziegler 2009). A mixture of morphologies were observed when spherulites were formed with fatty acids with similar chemical structure, or with the same fatty acid but under different processing conditions, which suggested that fatty acid chemical structure
is not solely responsible for the morphology of these spherulites (Fanta and others 2006). Two types of morphologies could mainly be differentiated as shown in Table 2.3.

Table 2.3. Spherulites formed from amylose inclusion complexes

<table>
<thead>
<tr>
<th></th>
<th>Large particles</th>
<th>Small particles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size and morphology</td>
<td>- Spherical/lobed particles</td>
<td>- Disc or torus shape particles</td>
</tr>
<tr>
<td></td>
<td>- 24 μm mean diameter</td>
<td>- 4.5 μm diameter</td>
</tr>
<tr>
<td></td>
<td>- Coarse and rough surface</td>
<td>- Depression in the center and spiral surface striation (Fanta and others 2002)</td>
</tr>
<tr>
<td></td>
<td>(Fanta and others 2002)</td>
<td></td>
</tr>
<tr>
<td>X-ray diffraction pattern</td>
<td>7₁ V-type diffraction pattern (Fanta and others 2002; Peterson and others 2005) which changed to a 6₁ conformation when redispersed in water (Shogren and others 2006)</td>
<td>6₁ V-type diffraction pattern (Fanta and others 2002; Peterson and others 2005)</td>
</tr>
<tr>
<td>Starch native lipid content (spherulites made from native corn starch)</td>
<td>- 52% linoleic</td>
<td>- 26% linoleic</td>
</tr>
<tr>
<td></td>
<td>- 29% palmitic</td>
<td>- 53% palmitic</td>
</tr>
<tr>
<td></td>
<td>- 13% oleic</td>
<td>- 13% oleic</td>
</tr>
<tr>
<td></td>
<td>- 3% linolenic</td>
<td>- 2% linolenic</td>
</tr>
<tr>
<td></td>
<td>- 3% stearic</td>
<td>- 6% stearic</td>
</tr>
<tr>
<td></td>
<td>Similar to native corn starch</td>
<td>Contained about twice as much the more linear palmitic acid as linoleic acid, which supports the 6₁ V-helical conformation (Peterson and others 2005).</td>
</tr>
<tr>
<td></td>
<td>The bulkier structure of linoleic acid supports the 7₁ V-helical conformation (Peterson and others 2005).</td>
<td></td>
</tr>
<tr>
<td>Temperature on slow cooling at which spherulites are formed</td>
<td>Forms first during cooling at a higher temperature (~86 °C). Further cooling did not significantly changed the appearance (Peterson and others 2005).</td>
<td>Forms during cooling at around 84 °C and their size and number increased when the dispersion was cooled to 82 °C. Further cooling did not significantly changed the appearance of these particles (Peterson and others 2005).</td>
</tr>
<tr>
<td>“Maltese cross” pattern when looked between cross polarizers</td>
<td>Strong Maltesse cross (Fanta and others 2002)</td>
<td>Strong Maltesse cross (Fanta and others 2002)</td>
</tr>
</tbody>
</table>

In addition to the other two morphologies, a third type of structure was also observed when the jet cooked common corn or high amylose maize starch dispersions were cooled at different conditions. Cooling faster (4 h vs 22 h) a common corn starch dispersion resulted in the formation of 1–2 μm spheres (Fanta and others 2005). Cooling slowly a high amylose maize starch dispersion while stirring produced trace amounts of
micron or sub-micron spherical particles, and in the absence of stirring, the amount of these particles increased (Fanta and others 2008). Quenching the dispersions to 25 °C in 7 min using an ice bath resulted in the formation of submicron spherical particles with a V_{61} helical conformation (Fanta and others 2008). These small spheres were also observed when mineral oil was added before processing the starch dispersion in the jet cooker (Fanta and others 2005).

Spherulites formed from amylose inclusion complexes have also been observed when maize starch was heated above 120 °C and stored at 75–95 °C (Davies and others 1980). Spherulitic crystallization induced by amylose complexation with geraniol (Heinemann and others 2005) and with δ-lactones (Heinemann and others 2003; Heinemann and others 2005) has also been reported. In these experiments, potato starch dispersions were heated to 121 °C, slowly cooled, and then flavors were added at 30 °C (Heinemann and others 2003; Heinemann and others 2005).

2.4.4. Hydrolysis of starch spherulites

Hydrolysis of starch spherulites appeared to depend on the type of crystalline structure: A-, B-, or V-type.

Enzymatic hydrolysis of A- and B-type spherulites made from short chain amylose (DP~20) has been studied using glucoamylase 1, α-amylase, and β-amylase (Williamson and others 1992). For A-type spherulites, glucoamylase 1 had the highest rate of hydrolysis followed by α-amylase and then by β-amylase. After hydrolysis with any of these three enzymes, A-type spherulites lost all their spherulitic structure. Based on X-ray diffraction patterns, it was suggested a C-type structure or the presence of a small amount of B-type crystalline material which was preferentially hydrolyzed (Williamson and others 1992). B-type spherulites made from short chain amylose were hydrolyzed slower than their A-type counterparts (Williamson and others 1992).
Planchot and others (1997) also reported a faster hydrolysis rate of A-type spherulites (1.3%/h) as compared to the rate of B-type spherulites (0.25%/h) after 30h hydrolysis. After this time, the degradation rate tended towards zero, and the final proportion of hydrolyzed starch represented 61% of A-type and 18% of B-type spherulites (Planchot and others 1997). Williamson and others (1992) suggested that one of the parameters for determining rate of hydrolysis is probably the accessible surface area. The uneven surface of A-type spherulites compared to the smooth surface of their B-type counterpart resulted in an initial larger accessible surface area, which dramatically increased once hydrolytic amylases broke the A-type spherulites into small pieces (Williamson and others 1992). Leloup and others (1991) observed that enzyme adsorption to the spherulite surface did not occur on acid treated spherulites, and proposed that the smoother surface of these spherulites prevented enzyme adsorption and consequently prevented hydrolysis. On the other hand, amylase adsorption to the hairy surface (i.e. dangling chains at the spherulite surface) of non-acid treated spherulites resulted in slow hydrolysis (1.65%) after 30 h (Leloup and others 1991). Helbert and others (1993) proposed that the high susceptibility to hydrolysis of these spherulites could be explained by the internal structure of the particles. A-type spherulites are formed by an assembly of long crystalline elements radiating from the center possibly forming long radial channels from which the enzyme can penetrate all the way to the center of the spherulite (Helbert and others 1993).

For B-type spherulites, β-amylase exhibited a slower rate of hydrolysis compared to the action of glucoamylase and α-amylase, which had the highest hydrolysis rate (Williamson and others 1992). B-type spherulites maintained their structure and birefringence after treatment with glucoamylase 1, suggesting that this enzyme acts only on the surface of the spherulites. However, after 158 h of hydrolysis with α-amylase and β-amylase, samples maintained their spherical morphology, but lost their birefringence suggesting a disruption of the order of the crystallites. Numerous small pits were observed after partial hydrolysis with β-amylase, while few large pits were observed when α-amylase was used. DSC and X-ray results suggested loss of crystallinity in B-
type spherulites, while in A-type spherulites, it appeared that the remaining fragments were composed by the same proportion of crystalline material (Williamson and others 1992).

In the case of V-type spherulites, Heinemann and others (2005) reported that starch flavor spherulites are slowly hydrolyzed by α-amylase at low concentrations (50 units/g dry starch), maintaining their structure after 2 h of hydrolysis. However, at high α-amylase concentration (2000 units/g dry starch), breakdown of spherulites occurs quickly within 30 min. This study suggested that hydrolysis of spherulites in the mouth is slow, while hydrolysis of amylose-flavor complexes in the gastrointestinal tract is fast, as evaluated by iodometric titration, DSC, and light microscopy (Heinemann and others 2005).

2.5. Encapsulation

2.5.1. Encapsulation in the food and pharmaceutical industry

Encapsulation involves the use of a capsule, wall material, membrane, carrier, or shell to entrap or coat a core material (Gibbs and others 1999; Risch 1995). In the food industry, materials that are used as encapsulating matrix include gum Arabic, dextrins, starches, maltodextrins, alginites, protein based materials, cyclodextrins, and lipid components (liposomes) (Gibbs and others 1999). Forms of encapsulation can vary as shown in Figure 2.4.

![Figure 2.4. Schematic representation of forms of microcapsules](Adapted from Gibbs and others 1999)
In the pharmaceutical industry, encapsulation has been used in order to enhance stability and to provide a medium to deliver drugs (Gibbs and others 1999). In the food industry, encapsulation of preservatives, antioxidants, Redox agents, acids, alkalis, buffers, colors, flavors, sweeteners, nutrients, cross-linking agents, enzymes, cells, or other materials is commonly used. Encapsulation of food ingredients provides them with protection from their environment, stability during processing, and controlled release capability (Reineccius 1995a). Encapsulation can also mask undesirable odor, color or taste, reduce volatility (Possell 2004), or provide an easier way to deliver the original liquid ingredient as a solid (Reineccius 1995a). Below are some examples of encapsulation of compounds that are of interest in the food and pharmaceutical industry.

**Encapsulation of vitamins**

Vitamins are essential micronutrients that are minor constituents in foods. Vitamins have various functions in the body including working as coenzymes or their precursors, as antioxidants, and as factors involved in genetic regulations and other reactions (Gregory III 1996). The main purpose of encapsulating vitamins is to extend their shelf life. Encapsulation can protect vitamins from oxidation or from reacting with other components present in the food (Schrooyen and others 2001).

Water soluble vitamins, such as vitamin C, can be encapsulated by spray cooling, spray chilling, and fluidized bed coating if the vitamin is going to be added to a solid food (Schrooyen and others 2001). The produced microcapsules are not soluble in water and will release the encapsulated material when the food product is heated above the melting temperature of the wall material (a fat or wax) (Kirby and others 1991; Schrooyen and others 2001). Liposome encapsulation has also been shown to increase the shelf life of vitamin C (Kirby and others 1991) and can be used to deliver vitamins in liquid food systems (Schrooyen and others 2001).
As compared to water soluble vitamins, lipid soluble vitamins, such as vitamin A, \( \beta \)-carotene and vitamins D, E, or K are easier to encapsulate. The most common method used for industrial encapsulation of oils-based compounds is spray drying. Gum Arabic and maltodextrins can be used as the matrix material. The produced spray dried powders are often used for the manufacture of tablets (Schrooyen and others 2001).

**Encapsulation of phytonutrients**

Phenolic compounds are naturally found as secondary metabolites in plants (Manach and others 2004). Based on the number of phenol rings and the linkage between these rings, these compounds can be classified as phenolic acids, flavonoids, stilbenes, and lignans (Manach and others 2004). The growing interest in some of these plant components are due to the antioxidant capacity and health benefits they are associated with. However, most phytonutrients found in fruits and vegetables are bitter, acrid or astringent, which makes the use of these compounds a challenge to functional food designers (Drewnowski and Gomez-Carneros 2000). For example, bitter and astringent catechins (Drewnowski and Gomez-Carneros 2000) are one of these polyphenolic compounds present in wine and tea, and have been related to cancer and cardiovascular disease prevention (Križ and others 2003).

Other phytonutrients of interest to be delivered in foods are plant sterols, also called phytosterols. Because of phytosterol’s similar structure to cholesterol (Thurnham 2007), these compounds compete with cholesterol for absorption in the intestine, resulting in a decrease in serum low density lipoprotein cholesterol levels (Lagarda and others 2006). Because of the lowering cholesterol effect of phytosterols, functional food products have been developed with high levels of phytosterols, including spreadable fats and yogurts (Päivi 2005).
Encapsulation of flavors and essential oils

Flavor encapsulation is commonly used to protect flavors against evaporation, reaction with other ingredients, or migration in a food (Madene and others 2006), or to control their release when slow release is desired (i.e. in chewing gums).

For many years, plant essential oils have been used for various purposes such as perfumery, flavoring, and preservation (Dang and others 2001). The antimicrobial activity of essential oils has been known (Conner 1993; Valero and Salmeron 2003), and their health benefits previously investigated (Carnesecchi and others 2004; Carnesecchi and others 2002; Youdim and Deans 1999, 2007). For example, geraniol is found in plant essential oils (Mourtzinos and others 2008) and it has been shown to have antimicrobial properties against Saccharomyces cerevisiae (Prashar and others 2003), anti-tumor properties (Yu and others 1995), and can also sensitize human colonic cancer cells to the effect of an anticancer drug (Carnesecchi and others 2004; Carnesecchi and others 2002).

Encapsulation of pharmaceuticals

Encapsulation of pharmaceuticals is also of great interest in order to increase the stability of these components and/or to control their release. Interest in delivering drugs (e.g. 5-aminosalicylic acid or indomethacin) directly to the colon via the oral route has increased in the past years (Rubinstein 2000). Delivering the drug to the active site increases drug bioavailability at the target site (Sinha and Kumria 2003) and may lower the administered dose reducing side effects (Sinha and Kumria 2001; Sinha and Kumria 2003). For the oral delivery of drugs targeted to the colon there are three basic methods: release of encapsulated drug caused by pH changes in the gastrointestinal tract, formulation with a determined time release after consumption, and degradation of the carrier by colonic bacteria (Ashford and others 1993). A combination of these methods can also be used (Sinha and Kumria 2002). Various polysaccharides have been evaluated.
to be used as part of the formulation of carriers for drugs targeted to the colon, including pectin (Ahrabi and others 2000; Ashford and others 1993, 1994; Sinha and Kumria 2001), xanthan gum (Sinha and Kumria 2001, 2002), guar gum (Gliko-Kabir and others 2000a; Gliko-Kabir and others 1998, 2000b; Krishnaiah and others 2002; Rama Prasad and others 1998; Sinha and Kumria 2001, 2002), chitosan (Sinha and Kumria 2001, 2002), and dextrins (Hovgaard and Brøndsted 1995). The use of amylose and ethylcellulose as a coating of the carrier has also been successful (Milojevic and others 1996a).

2.5.2. Encapsulation techniques

2.5.2.1. Spray drying

Spray drying is a widely used technique for encapsulation of many materials. It is one of the most common techniques for ingredient encapsulation, in particular for flavors (Gibbs and others 1999; Possell 2004; Risch 1995), oils, and vitamins (Possell 2004). The material to be encapsulated is homogenized together with the hydrated carrier material. This mixture is atomized in the spray dryer chamber into small droplets. The hot air quickly evaporates the water from the fine droplets, resulting in dry capsules (Gibbs and others 1999; Risch 1995). The carrier materials usually used in spray drying are modified starches, maltodextrins, gums (Gibbs and others 1999), soluble starch hydrolysates, and syrups (Forssell 2004). Gelatinized starch (Liu and others 2007) and small granular starches (Tari and others 2003; Whistler 1997; Zhao and Whistler 1994) have also been used to form spray-dried starch capsules. For example, Liu and others (2007) spray dried gelatinized sweet potato starch to produce drug capsules with 6-hour sustained release properties. One of the disadvantages of spray drying is that other components with emulsifying capacity, such as gum Arabic or water-soluble proteins need to be added if lipid soluble substances are encapsulated (Forssell 2004).
Advantages of this technique include lower production cost compared to other encapsulation techniques, ready availability of equipment, good protection of the encapsulated material, and availability of wide variety of wall material (Risch 1995). However, spray drying produces very fine powders which require extra agglomeration steps to improve solubility if the product is for a liquid application (Risch 1995).

2.5.2.2. Spray chilling or spray cooling

Similar to spray drying, the hydrated material and the material to be encapsulated are mixed and atomized into a chamber (Gibbs and others 1999; Risch 1995). However, in this case, cooled or chilled air is used instead of heated air and water is not evaporated (Gibbs and others 1999). Vegetable oil (melting point = 45–122 °C) and hydrogenated or fractionated vegetable oil (melting point = 32–42 °C) are usually used as the encapsulating material in spray cooling and spray chilling, respectively (Gibbs and others 1999; Risch 1995). These techniques are usually used for the encapsulation of vitamins, minerals, or acidulants (Gibbs and others 1999; Risch 1995).

2.5.2.3. Extrusion

In encapsulation by extrusion, the core material is mixed with a molten carbohydrate mass, and the mixture extruded into a dehydrating liquid. As a result, the carbohydrate mass hardens and the core material is entrapped in the carbohydrate matrix. Isopropyl alcohol is the most common dehydrating liquid used. The hard filaments or strands produced by this process are broken into small pieces which are finally dried (Risch 1995). Emulsifiers are often used when hydrophobic substances are encapsulated into the starch matrices to stabilize the mixture during processing (Yilmaz and others 2001). By this method, the wall material completely surrounds the encapsulated material.
After hardening of the carbohydrate mass, the remaining core material on the surface of the capsules is removed (Gibbs and others 1999; Risch 1995). Capsules produced by extrusion are relatively large with an excellent shelf-life (Risch 1995). This method is commonly used for visible flavor pieces, vitamin C, and colors (Gibbs and others 1999). Extrusion has also been used as a continuous process for encapsulation of biologically active agents, for example pesticides, in a starch matrix (Carr and others 1993; Doane 1993; Trimmell and others 1991). Starch and water are subjected to high shear and temperatures above the gelatinization temperature, after which the active component is added, and the mixture subjected to high shear to ensure a homogeneous distribution through the starch matrix. Release properties are determined by controlling processing parameters (Carr and others 1993).

2.5.2.4. Other techniques

Fluidized bed coating

Coating material is atomized over solid particles that are suspended in a temperature and humidity control chamber of an upward-moving stream of air (DeZarn 1995; Risch 1995). The length of time that particles stay in the chamber determines the amount of coating material applied on the particles (Risch 1995). Some applications of encapsulation by this technique include: separation of reactive components within a mixture, controlled release, reduction of hygroscopicity, and improvement of flowability (DeZarn 1995).

Liposome entrapment

Liposomes consist on a phospholipid-based membrane completely surrounding an aqueous phase (Reineccius 1995b; Risch 1995). They are spontaneously formed when phospholipids are dispersed in an aqueous media (Risch 1995). The size of these vesicles
range from few nanometers to micrometers (Reineccius 1995b). Aqueous or lipid soluble materials can be encapsulated by liposomes, but not materials that have both aqueous and lipid phases. Since most flavors have some solubility in both aqueous and lipid phases, they cannot be encapsulated in liposomes (Reineccius 1995b; Risch 1995).

**Coacervation**

The process of coacervation involves phase separation at different degrees of a polymeric aqueous solution by changing the physicochemical environment of the solution. The process of coacervation can be “simple” or “complex”. In simple coacervation, a single colloid is dispersed in a solution. Then, colloid chains are forced to come closer together and form the coacervate by adding a chemical with greater affinity for the solvent than for the colloid. In complex coacervation, coacervates are formed by the interaction of colloids with two opposite charges (Subirade and Chen 2008).

**Inclusion complexation**

Encapsulation by inclusion complexation is the only encapsulation technique that occurs at the molecular level. β-cyclodextrins are usually used as the encapsulating material. β-cyclodextrin consists of 7 glucose units linked by 1-4 linkages, which results in a central hydrophobic cavity and an outer hydrophilic surface. When cyclodextrins and the core materials are in solution, less polar molecules will move to the center of the cyclodextrin replacing the water molecule held in this cavity. The complex precipitates out of solution due to a decrease in solubility of the complex (Risch 1995). Complexation with cyclodextrins can be used to protect the core material (e.g. fat soluble vitamins, flavors) from oxidation, heat, light, to reduce volatility, or improve solubility of some compounds (Hedges and others 1995). Similar to cyclodextrins, amylose can also form inclusion complexes with certain molecules (See Section 2.3) and these inclusion complexes may have potential as delivery systems of bioactive compounds (Cohen and
others 2008; Lalush and others 2005; Lesmes and others 2008; Lesmes and others 2009; Zabar and others 2009). The ability of starch to form inclusion complexes with some flavors has also been suggested as a method for flavor encapsulation when slow release is desired (i.e. chewing gums) (Heinemann and others 2003).

**Rotational or centrifugal suspension separation**

In this method, the core particles are suspended in the coating liquid. The passage of the suspension over a rotating disk results in coated core particles which are then dried or chilled (Risch 1995; Sparks and others 1995).

### 2.5.3. Methods of release of core materials

**Diffusion control**

The release of the core materials is controlled by controlling the diffusion of the encapsulated material to the surface of the capsule. This release can be matrix-controlled or membrane-controlled release. In the first case, the release is controlled by the bulk capsule material, and in the latter, by a membrane covering the wall (Gibbs and others 1999; Reineccius 1995a).

The activity or chemical potential as related to the vapor pressure of the volatile substance on each side of the membrane is the real driving force of diffusion (Gibbs and others 1999; Reineccius 1995a). The solubility of the component in the matrix, and the permeability of the component through the matrix are factors affecting the diffusion (Gibbs and others 1999; Reineccius 1995a). The chemical nature, morphology and glass transition temperature of the membrane affect its permeability. Degree of swelling and cross-linking of the membrane also affect the diffusion of the core material: The greater the degree of swelling, and the lower the degree of cross-linking, the faster the diffusion.
In addition, if the matrix is permeable to the fluid surrounding the capsule, then the fluid may enter the matrix and leach the encapsulated material out of the capsule (Fan and Singh 1989).

**Release by degradation or erosion**

The encapsulating material undergoes surface erosion and the core material is released at a rate dependent on the rate of degradation. Membrane and matrix type capsules are used for this type of release, with the matrix type the more suitable. Usually when this type of release is observed, loss of physical integrity and collapse of the capsule follow the erosion of the matrix (Fan and Singh 1989).

**Pressure activated release**

In the case of pressure-activated release, it is desired that the core material is not released until the membrane is ruptured. This type of release is used in carbonless paper and “scratch and sniff” products. The application of this method in the food industry is limited, and more applicable to food packaging than direct contact with food. For example, if aroma release is desired every time the consumer opens a jar of product, then the sealing surface of the jar can be coated with a rupturable aroma (Reineccius 1995a).

**Solvent-activated release**

This method is the most common release method used in the food industry. The core material is quickly released when the capsule is completely dissolved, or the release of the encapsulated material can begin or be enhanced by the swelling of the capsule (Reineccius 1995a). When the release is by swelling, the matrix absorbs the solvent from the surrounding, resulting in swelling of the encapsulating matrix facilitating the diffusion of the core material outside the capsule (Fan and Singh 1989).
**Osmotically controlled release**

In osmotically controlled release, the core material takes up the solvent, and as a result, the capsule swells and burst releasing the encapsulated material (Reineccius 1995a). The solvent is transported from the environment into the core by permeation (Fan and Singh 1989).

**pH sensitive release**

This type of release is based on the destabilization of the wall material by pH changes. (Reineccius 1995a).

**Melting activated release**

The core material is released when the capsule wall or the material coating the capsule wall melts. To achieve this type of release, spray cooling or spray chilling, two fluid nozzle extrusion, or secondary coating techniques are used. Numerous food applications require heating of the product immediately prior to use, so ingredients can stay protected until then. Some of these ingredients include nutrients, flavorings, and leavening (Reineccius 1995a).

2.6. **Starch based microencapsulation**

Starch and starch based ingredients (e.g. modified starches, maltodextrins and cyclodextrins) are widely used for encapsulation purposes (Madene and others 2006), due to their low cost, availability, biodegradability, food grade, and the fact that they can be easily modified (Öngen and others 2002).
Spray drying and extrusion are the most common processing techniques to produce starch-based microcapsules (Forssell 2004). These processes produce a carbohydrate matrix that physically entraps the core material (Zeller and others 1998) resulting in a matrix-type capsule (Forssell 2004).

Other encapsulation methods have been used to produce starch-based capsules. However, these methods are mostly batch processes on a pilot-plant or laboratory scale. For example, starch capsules have been produced by gelatinizing starch by jet cooking, mixing the core material, drying the starch mass in a forced-air oven and grinding the dry material to produce fine particles (Wing and others 1988). Other researchers have produced starch capsules by letting the starch slowly crystallize and precipitate after jet cooking in the presence of the core material (Heinemann and others 2003; Heinemann and others 2005). Starch capsules have also been produced by increasing the porosity or creating cavities in the native granular structure to be filled with a variety of compounds (Forssell and others 2003; Ishii and others 1999; Korus and others 2003; Lii and others 2003; Myllärinen and others 1999; Whistler 1991).

Molecular encapsulation of guest molecules in inclusion complexes with cyclodextrins is well known. The formation of inclusion complexes of a wide variety of small molecules and starch has also been known for many years. However, only recently, have these starch inclusion complexes been seriously investigated as a delivery system of bioactive compounds (Cohen and others 2008; Lalush and others 2005; Lesmes and others 2008; Lesmes and others 2009; Zabar and others 2009). Two methods are commonly used to induce the formation of starch-inclusion complexes (Lesmes and others 2008). One method comprises the dissolution of starch in hot DMSO, addition of the guest molecule, followed by slow precipitation of the complexes after water addition. In the second method, starch is solubilized in an alkali solution, and after the guest compound is added, complexes are precipitated by the addition of acid.
Recently, emulsion techniques have been used to produce starch-based micro- (Elfstrand and others 2007a; Elfstrand and others 2007b; Elfstrand and others 2004) and nano-capsules as carriers of active substances (Santander-Ortega and others 2009; Zhai and others 2008). For the production of the starch microparticle Biosphere®, intended for subcutaneous injections, waxy starch is emulsified in an aqueous two-phase system containing polyethylene glycol (Elfstrand and others 2007a). The microspheres are stabilized by crystallizing the starch matrix. Some of the factors that affect the starch matrix quality include the amount of crystallites and ordered structure, and their thermal stability. These factors are affected by the time and preset temperatures that the starch emulsion is held during processing (Elfstrand and others 2007a).

Nanocapsules made of propyl-starch derivatives were produced using an oil-in-water emulsion diffusion technique. The propyl-starch derivative was solubilized in ethyl acetate, the solution was combined with an aqueous phase containing polyvinyl alcohol, and emulsified using a high speed homogenizer. After emulsification, complete diffusion of the organic solvent to the aqueous phase was forced by adding 10 ml of water. Finally, the stable nanoparticles were obtained by evaporating the organic solvent under vacuum at 35 °C (Santander-Ortega and others 2009).

Nanoparticles containing polyoxomelates, which are clusters of early transition metal oxygen anions known for their antiviral and antitumor properties (Rhule and others 1998), were produced using a water-in oil emulsion method (Zhai and others 2008). A 15% starch solution was prepared by heating soluble starch dispersion in a boiling water bath until the solution became transparent, and adding the polyoxomelate after cooling. The aqueous solution was transferred drop-wise to an oil phase composed of 50/50 toluene/chloroform and a surfactant. The system was stirred until an emulsion formed. In order to achieve smaller nanoparticles, the emulsion was treated using ultrasound for 30 min. A specific amount of phosphoryl chloride, a cross-linking agent, was then added and the system was stirred for 1 h, after which the system was left undisturbed to obtain two phases. The aqueous phase was separated from the organic phase and washed with
acetone and ethanol to obtain the polyoxomelate-starch nanoparticles (Zhai and others 2008).

2.6.1. Starch polymers and granules in encapsulation

In addition to the advantages of starch described above (availability, biodegradability, food grade, etc.), its physicochemical properties, including retrogradation (recrystallization), film forming ability, complex formation, and resistance to various degrees towards amylase hydrolysis, make starch a promising material for delivery systems. Consequently, the use of starch polymers and granules as encapsulating material has recently garnered attention.

A wide variety of compounds have been encapsulated in starch matrices. A common method to produce these capsules comprises adding the active compound into a gelatinized starch solution and, after mixing, the mixture is dried and ground to form the final particles (Öngen and others 2002; Wing and others 1987, 1988).

Wing and others (1987) produced pearl corn starch capsules containing an herbicide by jet-cooking the starch at high temperatures (90 °C to 143 °C), followed by addition of the active component, drying, and grinding of the mixture. The resultant product had slow release properties, which were greatly affected by the rate and extent of starch retrogradation (Wing and others 1987). Because of the ability of amylose to retrograde, higher amylose content resulted in a slower release rate (Wing and others 1988). Retrogradation properties of amyllopectin have also been reported to modify the release properties of starch capsules. Yoon and others (2009) retrograded waxy maize starch gels by exposing the gels to 4 °C or to temperature cycles of 4 °C and 30 °C for 2 days at each temperature for a total time of 8 days. The 4/30 cycle treatment resulted in a more compact matrix structure, with higher resistance to amylase hydrolysis and decreased swelling, which produced capsules that effectively delayed the release of the drug theophylline.
Kneading, a thermo-mechanical process, has been used as a method to encapsulate a thermostable α-amylase and a model volatile compound, diacetyl, in a starch matrix (Öngen and others 2002). Starch hydrolysis by amylase was suggested as the mechanism triggering the release of the encapsulated compound (Yilmaz and others 2002). The enzyme was not active at low humidity conditions and could be activated in a humid environment of 90% relative humidity, maintaining 90% of its enzyme activity (Öngen and others 2002). In addition to amylase, other enzymes such as lactase, invertase, and catalase, can be immobilized in a starch gel. After starch gelatinization, the enzymes are added upon cooling, and the gelled mixture is then extruded into strands that are later dried and broken into pieces (Muettegeert and others 1983).

Kapusniak and Tomasik (2006) encapsulated lipids (oleic, linoleic, and stearic acid) in starch by short microwave heating of granular potato, waxy corn, and tapioca starches in the presence of lipids. These authors showed that with this method, guest molecules did not react with starch and therefore, entrapment was not due to the formation of inclusion complexes.

Granular starches have been used as carriers of various compounds. Starch granules are pre-treated to form empty domains inside the granules, in which guest molecules can be trapped. Previous studies have claimed that swelling potato starch granules by soaking them in water or a saturated NaCl aqueous solution for an extended period of time (up to 48 h) causes the loss of the amorphous material, leaving empty spaces in the granular structure (Korus and others 2003; Lii and others 2003). Korus and others (2003) used these potato starch capsules for aroma encapsulation. In addition to their encapsulating ability, Lii and others (2003) suggested that this type of potato starch capsule may have higher proportion of dietary fiber content because of the higher proportion of crystalline material in the potato starch structure after swelling.
The starch granule structure can also be made porous by partial hydrolysis (Forssell and others 2003; Ishii and others 1999; Myllärinen and others 1999; Whistler 1991) or by the action of amylases (Kobayashi and others 1993; Myllärinen and others 1999; Whistler 1991; Zhao and others 1996). The resultant porous structure can absorb aromatic components (Kobayashi and others 1993) or other hydrophobic substances (Kobayashi and others 1995; Zhao and others 1996), or be filled with various compounds including polypeptides or proteins (Forssell and others 2003; Myllärinen and others 1999), drugs, fertilizers, pigments, oils, and food additives (Ishii and others 1999). Filled granules are then coated with a biopolymer film such as starch or amylose (Forssell and others 2003; Myllärinen and others 1999) or other coating agent (Ishii and others 1999).

Partially hydrolyzed starch granules coated with biopolymer films have been proposed as a potential carrier for microorganisms or enzymes protecting them during storage or during their transit through the intestine (Myllärinen and others 1999). Another method to deliver viable microorganisms, such as probiotic bacteria, to the large intestine is the adhesion of the microorganisms to the surface of the granule (Crittenden and others 2001; Wang and others 1999). Adhesion of some Bifidobacterium strains to high amylose maize starch granules protected living cells when exposed to low pH, bile acids and mice’s gastrointestinal tract conditions (Wang and others 1999).

Small starch granules have been used to form spherical porous aggregates that can serve as carriers of food ingredients (Forssell 2004; Tari and others 2003; Zhao and Whistler 1994). Rice, amaranth, quinoa, and colocasia starch granules, or the small size fraction of other starches such as wheat starch, can be used to form these aggregates. Aggregates are produced by spray drying a 20–30% aqueous dispersion of starch in the presence of a proper binder (0.1–1%) (Tari and others 2003; Zhao and Whistler 1994). Proteins or water soluble polysaccharides, such as gelatin, gum Arabic, carboxymethyl cellulose, and carrageenan can be used as the bonding material (Tari and others 2003; Whistler 1997; Zhao and Whistler 1994). The dispersion is spray dried using processing conditions that avoid starch gelatinization. The core material can be incorporated either before or after spray drying. In the latter case, the core material is added with the help of
a low boiling point solvent, which is then evaporated (Whistler 1997; Zhao and Whistler 1994). Zhao and Whistler (1994) reported that these aggregates can retain considerable peppermint oil (up to 48%) even after a brief ethanol washing and drying. Tari and others (2003) successfully encapsulated vanillin in these type of starch aggregates. The performance of the aggregates as encapsulating material can be improved by coating them with a polymer after addition of the active component (Whistler 1997; Zhao and Whistler 1994).

The ability of starch and amylose to form a film has been used to improve the performance of delivery systems. For example, amylose-based (McConnell and others 2007; Milojevic and others 1996a, 1996b) and high amylose maize starch-based films (Freire and others 2009b; Podczeck and Freire 2008) have been used to coat pellets for colon specific drug delivery. A mixture of amylose or starch with ethylcellulose was necessary to avoid swelling of the film (Milojevic and others 1996a, 1996b). The use of amylose or starch/ethylcellulose films prevented the release of the model drug, 5-aminosalicylic acid, under simulated stomach and small intestine conditions, giving rise to their potential use for colon specific delivery systems (Milojevic and others 1996a, 1996b; Podczeck and Freire 2008). Milojevic and others (1996a) suggested that the microstructure of the glassy amylose film provides the resistance towards pancreatic amylase hydrolysis. However, Freire and others (2009a) attributed these results to the resistance of retrograded starch to various amylases. Further studies showed that pellets coated with the amylose/ethylcellulose film release the drug under simulated colonic conditions (McConnell and others 2007). In-vivo evaluation of amylose/ethylcellulose coated pellets showed that the encapsulated drug was detected only upon arrival of the pellets in the colon (McConnell and others 2008). Most of the encapsulated drug (92%) was released in the colon, while the remaining was disposed of the fecal material (Basit and others 2009). These amylose/ethylcellulose coated pellets provided better drug delivery to the colon as compared to pellets coated by a pH triggered-film (McConnell and others 2008).
2.6.2. Starch inclusion complexes as molecular nanoencapsulants

The ability of starch to form these inclusion complexes has been used for molecular encapsulation with controlled released properties for flavors (Heinemann and others 2005) and nutraceuticals (Cohen and others 2008; Lalush and others 2005; Lesmes and others 2008; Lesmes and others 2009; Yang and others 2009). Amylose-lipid complexes have also been suggested as a mechanism to control release of lipids during starch gelatinization and pasting (Gelders and others 2006).

Heinemann and others (2005) formed inclusion complexes of γ-nonalactone, δ-dodecalactone and geraniol with potato starch. These complexes induced spherulitic crystallization of the starch. Their results suggested that these spherulites might be slowly hydrolyzed in the mouth, while they would be completely hydrolyzed in the gastrointestinal tract. The same authors proposed that spherulitic amylose-flavor complexes might be useful in food systems where a controlled flavor release is desired, for example in chewing gums.

Lalush and others (2005) formed amylose inclusion complexes with conjugated linoleic acid (CLA) and suggested these complexes as molecular nanocapsules for protection and delivery of CLA. In their study, complexes were produced using two different methods: DMSO/water solution (Eliasson and Krog 1985) and KOH/HCl solution (Karkalas and others 1995). The formation of inclusion complexes provided protection to the CLA against oxidation during storage, and retained the fatty acid in simulated stomach conditions (Lalush and others 2005; Yang and others 2009). CLA was released by amylase and pancreatin digestion of the complexes suggesting that the release of CLA would occur in the small intestine. Therefore, lipid-inclusion complexes were suggested as carriers of polyunsaturated fatty acids with controlled release properties to the small intestine (Lalush and others 2005).
Yang and others (2009) compared the performance of amylose-CLA and β-cyclodextrin-CLA complexes as delivery systems. Amylose-CLA complexes protected the CLA against oxidation better than their β-cyclodextrin counterpart. CLA was not released from the amylose inclusion complex under simulated stomach conditions for 2 h, but it was almost completely released (95%) in 15 h under simulated small intestine conditions. On the other hand, approximately 11% CLA was released from β-cyclodextrin complexes in simulated stomach conditions, while only 16% of the CLA was released under small intestine conditions. These results suggested that inclusion complexes of CLA with amylose, as compared to those with β-cyclodextrin, provide a better protection of the lipid against oxidation and passage through the stomach, while it will be released in the small intestine where it is absorbed (Yang and others 2009).

Cohen and others (2008) successfully formed inclusion complexes of amylose and high amylose maize starch with the isoflavone genistein by the KOH/HCl solution method. The genistein content of these complexes was 11 mg and 9 mg per 100 mg of complex for amylose and high amylose starch complexes, respectively. Based on the X-ray diffraction patterns, the complexes were of the V_{6III} type suggesting that the location of the guest molecule is between helices. Complexes were stable to various pH conditions and at 30 °C and 50 °C. High retention of genistein in the complexes was observed in simulated stomach conditions, but upon digestion in pancreatin solution, an average of 42% and 23% of genistein was released from high amylose maize starch and amylose complexes, respectively.

The preparation of inclusion complexes has been limited to batch processes, and the development of a continuous process would be of great industrial relevance (Lesmes and others 2008). Lesmes and others (2008) evaluated a continuous dual feed homogenization process to prepare starch inclusion complexes for controlled release of nutrients. These authors used stearic acid as a model guest molecule. The process comprised dissolution of high amylose corn starch or common corn starch in a hot alkali solution followed by mixing with a KOH stearic acid solution. The alkali solution is
pressurized through a homogenizer together with a phosphoric acid solution. This process results in rapid complexation. Upon hydrolysis of the complexes with pancreatic amylases for 24 h at 37 °C, approximately 10% and 60% of the stearic acid was released from high amylose maize starch and common corn starch, respectively. In addition to offering the advantage of a continuous process, this method overcame some disadvantages of producing complexes in batches: larger particle size, particle aggregation, and prolonged production time.

2.6.3. Starch hydrolysates as encapsulating material

Starch hydrolysis products, such as maltodextrins and syrups, are water soluble products widely used for encapsulation of flavors, some vitamins, and oils. However, when the core material is hydrophobic, these hydrolysates need to be combined with other components with emulsifying capacity such as gum Arabic or water soluble proteins. The bland flavor, high water solubility, low viscosity in solution, and low cost have made starch hydrolysates highly favored as microencapsulation matrices. Another advantage of these hydrolysates is that after spray drying, the resultant matrix is an amorphous glass, which provides a better protection to prevent evaporation or harmful reactions of the encapsulated material. However, these microcapsules are highly hygroscopic and caking of the powder can occur resulting in powder agglomeration (Forssell 2004).

In addition to their use as the encapsulating matrix, dextrins have also been used to improve the performance of other carriers. Similar to the use of starch or amylose films to coat pellets targeting drug delivery to the colon, dextrin-based films can also be used to coat colon specific drug carriers. Karrout and others (2009) coated 5-aminosalicylic acid-loaded beads with a branched dextrin/ethylcellulose film. These authors showed that the drug was not released in simulating upper gastrointestinal tract conditions, but was completely released after immediate contact with fecal samples of inflammatory bowel disease patients.
Pre-treated waxy corn starch has been used to produce starch microspheres for drug delivery as a subcutaneous injection (Elfstrand and others 2007a; Elfstrand and others 2007b; Elfstrand and others 2009). The pre-treatment of the starch consisted in acid hydrolysis and high pressure homogenization. The purpose of this pre-treatment was to obtain a product without any granular or crystalline order, while maintaining similar chain properties such as degree of polymerization, degree of branching, and unit chain length as the original starch (Elfstrand and others 2009).

2.6.4. Starch derivatives as encapsulating material

To overcome the disadvantage of adding emulsifiers to maltodextrins for encapsulation of lipophilic substances, starch can be modified by adding to the backbone a lipophilic group (Forssell 2004). Octenyl succinate at a level of 0.02 degree of substitution is the lipophilic component used to modify these starches (Reineccius 1991). Some advantages of using this modified starch as encapsulating matrix during spray drying is the good volatile retention and the possibility of using higher feed solid content as compared to gum Acacia. However, these modified starches do not provide good stability against oxidation during storage (Reineccius 1991).

Starch sodium octenyl succinate has been used to produce nanoparticles (~100nm) of vitamin E for use in a beverage (Chen and Wagner 2004). The starch was dissolved in distilled water at 80 °C, and after cooling the solution to 30 °C, vitamin E acetate was slowly added, and the mixture was initially homogenized with a high shear mixer to achieve droplet sizes below 1.5 μm. The emulsion was then homogenized by ultra high pressure. Various homogenization cycles were performed until the target particle size was obtained. Following homogenization, the emulsion was spray dried to produce a powder containing 15% vitamin E acetate that was stable in the beverage and did not affect its appearance.
Cyclodextrins are cyclic molecules made of glucose units and produced enzymatically from starch. These molecules have a hydrophobic internal cavity in which hydrophobic molecules can be entrapped (Forssell 2004). These entrapped molecules can be totally or partially included in the internal cavity of the cyclodextrin (Madene and others 2006). The protection against degradation and loss that cyclodextrins offer to the guest molecules, together with the improvement in processing performance, handling, and storage of food ingredients make cyclodextrins an attractive encapsulating material. However, loading is limited to 6–15%, and only guest molecules that are small enough to fit in the central cavity can be encapsulated (Forssell 2004).

Recently, starch-based micro- (Elfstrand and others 2007a; Elfstrand and others 2007b) and nano-capsules (Santander-Ortega and others 2009; Zhai and others 2008) have been produced as carriers of active substances for nasal (Björk and Edman 1988; Edman and others 1992; Mao and others 2004) oral, intramuscular (Heritage and others 1996; Mundargi and others 2008; Rydell and others 2005; Sturesson and Degling Wikingsson 2000), subcutaneous (Elfstrand and others 2007a; Elfstrand and others 2007b; Elfstrand and others 2004) and transdermal delivery (Santander-Ortega and others 2009). Some of the studies have used starch cross-linked with epichlorohydrin, while others have prepared the capsules by polymerization of acrylated starch in an oil-in water emulsion (Elfstrand and others 2009).

Modification of starch by treating high amylose starch (70% amylose) with epichlorohydrin produces a new polymeric material usually referred as cross-linked amylose. This material has been used as a drug carrier with controlled release properties (Dumoulin and others 1999; Mundargi and others 2008). Direct compression of the dry cross-linked amylose together with the drug, produces tablets that can have a linear release kinetic for 15–24 h. The drug release time increases with the degree of amylose cross-linking. Depending on the degree of cross-linking, this material can still be hydrolyzed by α-amylase (Dumoulin and others 1999). Further modification of epichlorohydrin cross-linked starch by various levels of substitution with carboxymethyl
or aminoethyl groups has also been evaluated for sustained release properties. The degree of modification to achieve a satisfactory sustained release was affected by the proportion and structure of amylose and amylopectin from the different botanical sources (Onofre and others 2009). Cross-linking of starch or dextrans with epichlorohydrin is also used to produce degradable starch microspheres for nasal drug delivery systems. The drug is loaded into the microsphere by adding an aqueous solution of the drug to the microspheres to form a gel, which is then freeze dried (Pereswetoff-Morath 1998). These degradable starch microspheres have been used for delivery of insulin improving its absorption (Björk and Edman 1988).

Tan and others (2009) precipitated starch acetate nanospheres by dropwise addition of water into an acetone solution of starch acetate. Because of the presence of relatively non-polar internal domains, these nanospheres were suggested to have potential for hydrophobic drug encapsulation. Starch nanoparticles loaded with polyoxometalates were produced using a water-in-oil emulsion technique. The resultant round nanoparticles (50–100 nm) contained 12% of polyoxometalates and provided higher drug stability and antitumor activity (Zhai and others 2008). Santander and others (2009) used an oil-in-water emulsion method to produce starch-based nanoparticles for transdermal drug delivery. These authors used propyl-starch derivatives for the production of these nanoparticles and loaded them with flufenamic acid, testosterone, or caffeine as drug models. The release profile of the loaded compounds showed an initial linear release without a burst effect.

2.6.5. Starch spherulites as encapsulating materials

As described in Section 2.4, spherulites can result from either the rapid crystallization of starch in the absence (or near absence) of lipid (Creek and others 2007; Creek and others 2006; Nordmark and Ziegler 2002a, 2002b; Ziegler and others 2005; Ziegler and others 2003) or the slow crystallization of starch in the presence of guest molecules that form starch inclusion compounds (Bhosale and Ziegler 2009; Byars and
Spherulites demonstrating V-type crystallinity can form various particle morphologies depending on environmental factors such as the precise nature of the thermal treatment, the starch concentration, the pH, the guest molecule and mechanical shear (Byars and others 2003, 2006; Fanta and others 2002; Fanta and others 2005; Fanta and others 2006, 2008; Fanta and others 1999; Heinemann and others 2005; Peterson and others 2005; Shogren and others 2006). Spherical, lobed (either 2 or 4 lobes), sintered (“snowball”), torus/disk, and bowtie shaped particles are among the morphologies observed, which may vary greatly in size and susceptibility to enzymatic degradation, and which may form the basis for controlled or targeted release of bioactives.

Spherulites could be employed for delivery of bioactives in much the same way as native starch granules. For example, successful matrix encapsulation of fluorescein dye in starch spherulites via confocal scanning laser microscopy has been observed (Ziegler, personal communication, May 14, 2008), and some spherulites possess a central cavity (Nordmark and Ziegler 2002b) that could be filled with active compounds to produce a core-and-shell capsule. However, unlike granules, spherulites have the potential for controlling properties such as release rate and location through control of their size, crystal type, crystallinity and enzyme resistance. McConnell and others (2008) demonstrated that a bacterially-triggered delivery system comprising amylose and ethylcellulose provided improved colonic delivery of theophylline vis-à-vis a pH-responsive polymer. Spherulites resistant to α-amylase may provide similar targeted release. Furthermore, they could be made safe for intravenous use.
2.6.6. Encapsulation of bioactives using ternary complexes

The formation of amylose guest inclusion complexes is limited by the size, shape, and polarity of the guest molecule, and while many bioactives may form such complexes, other molecules of interest may not. Little data exists on amylose-guest inclusion complexation in ternary systems, i.e. amylose-guest 1-guest 2. Rutschmann and Solms (1990) observed decreased binding of menthone in the presence of monostearate. Wulff and others (2005) concluded that two compounds could be complexed within the same helix and that it was possible to obtain a mixed complex of three different molecules. Tapanapunnitikul and others (2008) observed that binding of low-solubility flavor compounds was enhanced in the presence of native lipids and suggested that ternary co-inclusion complexes of starch-lipid-flavor may be formed.

β-carotene, lycopene and astaxanthin are all carotenoids with limited water solubility, susceptible to oxidation and have low bioavailability. Attempts to form inclusion complexes between these compounds and amylose using the DMSO method (Karkalas and others 1995) failed, likely due to the very low solubility of the guest molecules in aqueous DMSO. However, when the ternary mixtures amylose-ascorbyl palmitate-carotenoid were precipitated from hot 95% aqueous DMSO with excess water, V-complexes were formed and the carotenoid was partitioned into the starch matrix. It is likely that the inclusion complex was formed between the amylose and the alkyl chain of the palmitate ester, and that the carotenoid was "emulsified" into the matrix. However, complexation between amylose and carotenoid cannot be completely ruled out. These ternary matrices resulted in a significantly greater stability of the carotenoid to oxidation, even when compared to a physical mixture of the three components, probably due to the intimate association of the ascorbyl head group and the carotenoid. The release of carotenoid from the starch matrix was facilitated by enzymatic hydrolysis with pancreatin. This approach was generalized by replacing amylose with high-amylose starches, replacing ascorbyl palmitate with other emulsifiers including Tween 80, and incorporating other bioactives such as retinoic acid and phytosterols.
Some bioactives of interest, e.g. epigallocatechin-3-gallate (EGCG), are too water soluble to effectively partition into the starch matrix. For these systems it may be necessary to tailor the head group of the “emulsifying” complex-forming compound to provide specific interactions that attract the active. Another strategy to overcome this limitation would be the development of a prodrug of the bioactive that is less water soluble. Lambert and others (2006) peracetylated EGCG, reducing its biotransformation in the small intestine, and making it more bioavailable. Ester-based prodrugs are classical means to improve bioavailability and reduce toxicity. Fatty acid esters of these compounds may also partition better into amyllose-guest inclusion complexes or form complexes outright.

2.6.7. Modified starches as prodrugs

Matrix materials, including starch, are often used for the protection of sensitive bioactives as they transit the harsh environment of the stomach on the way to the intestine where they can be absorbed. However, it may also be desirable to protect the stomach from the side effects of some drugs, e.g. non-steroidal anti-inflammatory drugs (NSAIDs) that can promote gastric ulcers and perforations. One strategy is the use of prodrugs. Compounds that are not drugs themselves, have fewer side effects than the active compound, but are metabolized to the bioactive compound at the appropriate place and time.

Cai and others (2008) esterified indomethacin, a typical model NSAID, to amyllose to a level of 21.6 mg/g. The release of indomethacin was followed \textit{in vitro} under conditions simulating the stomach, small intestine and the colon. Indomethacin was hardly released in the simulated stomach medium, but was released on exposure to \(\alpha\)-amylase and lipase contained in the simulated small intestine and colonic media.
The short chain fatty acids (SCFA), acetate, propionate, and butyrate, have a role in the maintenance of human bowel health. Butyrate may be protective against large bowel cancer and is produced by colonic fermentation of carbohydrates (e.g. starch) that resist digestion in the small intestine. While increasing SCFA in the colon through increased consumption of fiber, especially resistant starch, may help many individuals, the microflora of others may have limited capacity to produced butyrate or may not tolerate dietary fiber for reasons such as increased flatulence. Therefore, Clarke and others (2008) compared colonic butyrate levels and azoxymethane-induced colorectal cancer in rats fed with either normal maize starch, normal maize starch plus tributyrin, high-amylose maize starch (an enzyme resistant starch) or the “prodrug” butyrated high amylose maize starch. The highest concentrations of butyrate in large bowel digesta and the lowest proportion of rats with tumors resulted from a diet with butyrated high amylose maize starch. The investigators concluded that SCFA esterified to a starch carrier was liberated by bacterial enzymes and utilized by gut microbes or colonocytes.

2.7. References


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Chapter 3
EFFECT OF STARCH FRACTIONS ON SPHERULITE FORMATION AND MICROSTRUCTURE

3.1. Introduction

Spherulites have been described as approximately radially-symmetric semicrystalline structures formed by crystal lamellae or fibers. When these structures are viewed between cross polarizers, they exhibit a characteristic “Maltese cross” extinction pattern (Creek and others 2006). Two types of spherulitic crystallization from native starches have been reported. In one case, spherulitic crystallization is induced by the formation of amylose inclusion complexes with fatty acids, and small torus-shape and large spherical/lobed particles are mainly formed. To obtain this type of spherulite, an aqueous starch dispersion is heated to 140 °C in the presence of lipids followed by slow cooling (Fanta and others 2002; Fanta and others 2005; Fanta and others 2006; Peterson and others 2005; Shogren and others 2006). The second type of spherulite has a spherical morphology and is formed when an aqueous starch dispersion is heated above 170°C followed by rapid cooling (Nordmark and Ziegler 2002a, 2002b; Ziegler and others 2005; Ziegler and others 2003). The formation of these spherical particles does not require the presence of amylose inclusion complexes (Nordmark and Ziegler 2002a) and is the focus of this chapter.

Ziegler and others (2003) reported that a heat treatment above 170 °C and fast cooling were necessary for the formation of well developed spherulites. Creek and others (2006) hypothesized that this temperature is necessary to go through a helix → coil transition in order to avoid gel formation during cooling caused by the presence of a helical nuclei. These authors proposed that during heating, when the dispersion reaches around 70 °C, double helical crystallites “dissolve”, and by 130 °C thermal dissociation
of fixed network entanglements occurs to form a liquid crystalline phase. Then, between 160 °C and 180 °C, the solution becomes isotropic, which is necessary for spherulite formation on cooling (Creek and others 2006). During cooling, phase separation is induced, resulting in a polymer-poor phase, and a polymer-rich phase in which spherulitic crystallization occurs. At slow cooling rates, crystallization can occur before phase separation, which may result in network formation preventing demixing (Ziegler and others 2005).

Some structural features reported for spherulites made from high amylose maize starches (ae 70) are a radially oriented structure, and the presence of a central cavity and small holes away from the center (Nordmark and Ziegler 2002b). Creek (2007) also suggested a radially oriented layered structure for spherulites made from common corn amylose with various degree of polymerization (DP). Atomic force microscopy (AFM) images of the interior of spherulites have shown the presence of blocklets of 20–60 nm (Creek 2007; Suwanayuen 2009) similar in appearance to blocklets observed in granular starches (Creek 2007; Sujka and Jamroz 2009).

Spherulite formation depends on the starch source (Ziegler and others 2003). Nordmark and Ziegler (2002) studied the spherulitic crystallization of high amylose maize starch (ae 50 and ae 70), common corn starch (CCS), waxy maize starch, amylose from high amylose maize starch (ae 70), and amylopectin from waxy maize starch. These authors reported that spherulite formation is favored by linear material (amylose) and that spherulites were practically absent in amylopectin samples (waxy maize). Similarly, Creek and others (2006) reported that amylopectin from potato starch did not form spherulites when heating a 10% dispersion to 180 °C and cooling it to 10 °C at cooling rates varying from 1–250 °C/min. Nordmark and Ziegler (2002a) suggested that the lightly branched intermediate material may be ideal for spherulite formation. However, the effect of various proportions of starch fractions (amylose, amylopectin, and intermediate material) on spherulite formation and microstructure has not been studied. The objective of this study was to investigate the effect of amylose, amylopectin, and
intermediate material obtained from common corn starch, high amylose maize starch, and potato starch, on spherulite formation and its microstructure.

3.2. Materials and Methods

3.2.1. Materials

High amylose maize starch (Hylon VII) and CCS were supplied by National Starch and Chemical Company (Bridgewater, NJ, USA). Potato amyllose from Sigma-Aldrich, Inc (St. Louis, MO, USA) and potato amylopectin from Fluka Chemie (Buchs, Switzerland) were used. Dimethyl sulfoxide (DMSO), isoamyl alcohol and 1-butanol were obtained from Sigma-Aldrich, Inc (St. Louis, MO, USA).

3.2.2. Starch fractionation

Starch fractions, amylose, amylopectin, and intermediate material, from Hylon VII and CCS were obtained by differential alcohol precipitation using isoamyl alcohol and 1-butanol as described by Klucinec and Thompson (1998). First, 10 g of CCS or Hylon VII were dispersed in 200 mL of a 90% DMSO aqueous solution in a boiling water bath with constant stirring for 3 h. The mixture was divided in four 250 mL centrifuge bottles. Non-granular starch was precipitated by adding 4 volumes of ethanol to each bottle and recovered by centrifugation at 6500 g for 15 min at 4 °C. Pellets were washed with ethanol (75 mL/bottle) and centrifuged as described above. The washing procedure was repeated one more time with ethanol and one time with acetone. The precipitate was dried in an oven at 50 °C for 24 h and stored in a desiccator until used.

A sample of 2.5 g of non-granular starch was dispersed in 70 mL of a 90% DMSO aqueous solution with constant stirring for 3 h in a boiling water bath. After dispersion, the non-granular starch dispersion was transferred into a 1 L glass bottle with
screw cap, and 7 volumes of a 6% 1-butanol-6% isoamyl alcohol aqueous mixture were added. After mixing, samples were placed in a water bath at 95 °C for 1 h. Then, the water bath was turned off and the system was allowed to cool to 28 °C for approximately 18 h. The suspension was centrifuged at 10 000 g for 15 min at 4 °C. The supernatant was collected and the precipitate was resuspended in 70 mL of water (~1–2% starch concentration). Seven volumes (490 mL) of a 6% 1-butanol-6% isoamyl alcohol aqueous mixture were added, and the heating, cooling, and centrifugation steps described above were repeated. The supernatant was combined with the first supernatant and the precipitate resuspended in 70 mL of water, mixed with 7 volumes of the 6% 1-butanol-6% isoamyl alcohol mixture, heated, cooled and centrifuged as described above. The supernatant was collected together with the other two supernatants. The collection of these 1-butanol/isoamyl alcohol supernatants contained the amylopectin fraction.

The precipitate was redispersed in 70 mL of a 90% DMSO aqueous solution using a magnetic stirrer. Then 490 mL of a 6% 1-butanol aqueous mixture were added. The mixture was heated in a water bath at 95 °C for 1 h, cooled to 28 °C, and centrifuged as described above. The supernatant was collected which contained the intermediate material fraction.

The precipitate (amylose fraction) was dispersed in 50 mL of 90% DMSO aqueous solution, and precipitated with 4 volumes of ethanol, washed two times with ethanol and once with acetone, and dried as described above for the preparation of non-granular starch.

The supernatants containing the amylopectin and intermediate material were concentrated fivefold using a rotary evaporator at 50 °C and a condenser connected to a circulating bath at 5 °C. Amylopectin and intermediate material fractions were precipitated from the concentrated supernatants using 4 volumes of ethanol, washed two times with 50 mL ethanol and once with 50 mL acetone, dried in an oven at 50 °C for 24 h, and stored in a desiccator until used.
3.2.3. Sample preparation

Starch spherulites with various proportions of amylose, amylopectin and intermediate material from Hylon VII and CCS were prepared following a simple lattice mixture design with a degree of lattice of 3 and one center point (Figure 3.1). Starch spherulites with various proportions of potato amylose and potato amylopectin were also prepared (100:0, 80:20, 60:40, 40:60, 20:80, and 0:100). Spherulites were produced in a differential scanning calorimeter (DSC-7, Perkin-Elmer Instruments, Norwalk, CT, USA). Starch fractions were weighed in 60 μL stainless steel DSC pans, and water was added to obtain a 10% (w/w) dispersion. Pans were hermetically sealed and kept overnight at room temperature for moisture equilibration. Samples were heated from 20 °C to 180 °C at 10 °C/min, and immediately cooled to 10 °C at a rate of 25 °C/min. Samples were stored overnight in the sealed pans before further analysis.

Figure 3.1. Mixture design for the preparation of starch spherulites with various proportions of amylose:intermediate material:amylopectin.

3.2.4. Microscopy

Immediately after opening the pans, samples were mixed using a micro-spatula, and a small aliquot was observed using a BX41 light microscope equipped with polarizing filters (Olympus, Edgemont, PA, USA) connected to a digital camera (Spot
Insight QE camera, Diagnostic Instruments Inc., Sterling Heights, MI, USA). The remaining sample in the DSC pans were washed with water, then with ethanol, dried at room temperature, and stored in a desiccator until further analysis.

For scanning electron microscopy (SEM), dried samples were fractured using a mortar and pestle to separate some clumps formed during drying. A thin layer of sample was placed on aluminum stubs with conductive carbon tape and sputter coated with gold. Samples were observed using an environmental scanning electron microscope (Quanta 200 ESEM, FEI Company, Hillsboro, OR, USA) under low vacuum mode of 0.68 Torr and voltage of 15 kV.

For transmission electron microscopy (TEM), samples were stained using the periodic acid thiosemicarbazide silver method as described by Planchot and others (1995). With this method, samples are stained before embedding, so staining after sectioning is not necessary. By this method, the starch is oxidized to a low degree, and the silver ions react preferentially with the amorphous regions, which appear dark in the TEM images (Gallant and others 1997b). To ensure a more homogeneous distribution of the sample and avoid losing material during sample preparation, dried samples were collected in a microcentrifuge tube and mixed with 50 µL of a 3% (w/v) agar solution at 45 °C. The agar was solidified at room temperature, and then broken into small pieces using a needle. Samples were treated with 1 mL of a 1% periodic acid solution for 30 min at room temperature followed by centrifugation. The precipitate was washed three times with deionized water. Then, samples were treated with 1 mL of a supersaturated solution of semithiocarbazide for 24 h. The semithiocarbazide solution was removed and 1 mL of a 1% silver nitrate aqueous solution was added. Samples were kept in this solution for 4 days in dark conditions. Samples were washed three times with deionized water, followed by dehydration by a series of 50%, 70%, 90%, and 100% aqueous ethanol. Samples were treated with 1 mL of each ethanol solution for 5 min, then washed three times with electron microscopy grade ethanol for 5 min, and three times with acetone for 5 min. Samples were infiltrated in a 50/50 eponate resin/acetone mixture for
7 h, and then in a 75/25 eponate resin/acetone mixture for another 7 h. After infiltration, samples were embedded in 100% eponate resin for 7 h followed by resin polymerization at 60 °C for 16 h (Evans 2002). Thin sections (70–90 nm) of the embedded samples were obtained and observed using a JEOL 1200 EXII TEM (Peabody, MA, USA). Thick sections (200 nm) were collected on a glass slide and stained with toluidine blue and viewed using a light microscope. Thick sections (500 nm) were obtained for AFM.

AFM was performed on a Digital Dimension 3100 atomic force microscope (Veeco Instruments, Wodbury, NY, USA) in tapping mode. Images were analyzed using the WSxM Scanning Probe Microscopy Software (4.0 v.12.6 2008, Nanotec Electronica S. L., Madrid, Spain). Roughness of the central and outer regions of the spherulites were determined from 0.5 μm x 0.5 μm images of at least two spherulites using the roughness analysis tool of the WSxM software, and reported as roughness root mean square (RMS). The width and height of the outer region of at least 5 spherulites per sample were measured using the profile tool as shown in Figure 3.2. Three different line profile curves were generated for each spherulite. For each profile, two measurements of the width and height of the outer region were obtained (Figure 3.2). Blocklet size was also measured using the profile tool. For blocklet size, three line profile curves were generated (one horizontal and two diagonal lines) from at least 2 images per region (central and outer region) and all blocklets of each profile curve were measured. Figure 3.3 shows the measurement of the size of one blocklet. The average of at least 60 blocklets per region was reported for each sample.

3.2.5. Thermal analysis

After storing samples overnight in the sealed pans, samples were heated in a Thermal Advantage Q100 DSC (TA Instruments, New Castle, DE, USA) to 180 °C at 10 °C/min. The DSC was calibrated with indium, and an empty sample pan was used as a reference. The baseline was obtained by processing two empty pans using the same heating treatment and subtracted from the data. Data was analyzed using the TA...
Universal Analysis software (Universal Analysis 2000 v.4.2E, TA Instruments-Waters LLC, New Castle, DE, USA).

Figure 3.2. Measurement of width and height of the outer region of spherulites: a) AFM topographic image (scan size 10 μm x 10 μm) of an amylopectin spherulite, and b) line profile of line shown on topographic image.

Figure 3.3. Blocklet size measurement: a) AFM topographic image (scan size 0.5 μm x 0.5 μm) of the central region of an amylose spherulite and b) line profile of dashed line shown on topographic image.
3.2.6. Statistical analysis

The mixture design was analyzed using the general regression model tool of Statistica 6.1 software (StatSoft, Inc, Tulsa, OK, USA). Multiple comparisons of roughness, blocklet size, outer region width and height of starch spherulites made from Hylon VII amylose, amylopectin, and intermediate material were performed using the “Proc GLM” and “means” procedures and Tukey test for multiple comparisons of SAS 9.1 for Windows (SAS Institute Inc., Cary, NC, USA).

3.3. Results and Discussion

Microscopy

All treatments containing Hylon VII amylose, amylopectin or intermediate material formed spherulites with the characteristic Maltese cross when observed between cross polarizers (Figure 3.4). In samples made of CCS amylopectin (Figure 3.5) and potato amylopectin (Figure 3.6) spherulites could not be observed. However, they were formed in all samples containing at least 33% CCS amylose or intermediate material, or 20% potato amylose. Previous studies have also reported that waxy maize (i.e. amylopectin) (Nordmark and Ziegler 2002a) and potato amylopectin (Creek and others 2006) did not form spherulites. The longer chains of amylopectin from Hylon VII starch (average chain length 48.5 DP (Klucinec and Thompson 2002)) as compared to the chains of waxy maize (average chain length 23–24 DP (Hizukuri 1985; Klucinec and Thompson 2002)), common corn (average chain length 26–28 DP (Hizukuri 1985; Klucinec and Thompson 2002)) and potato amylopectin (average chain length 34 DP (Hizukuri 1985)) could be responsible for the observed difference. Other factors such as chain length distribution or degree of branching may also be responsible for spherulitic crystallization of Hylon VII amylopectin. For example, Hylon VII amylopectin has a
Figure 3.4. Morphology of spherulites made with various proportions of Hylon VII amylose, intermediate material, and amylopectin observed under brightfield (left) and polarized (right) illumination. Scale bars = 10 μm.
Figure 3.5. Morphology of spherulites made with various proportions of CCS amylose, intermediate material, and amylopectin observed under brightfield (left) and polarized (right) illumination. Scale bars = 10 μm.
Figure 3.6. Spherulites made with various proportions of potato amylose and amylopectin observed under brightfield (top) and polarized (bottom) illumination. Scale bars = 20 μm.
higher proportion of longer to shorter chains as compared to common corn, waxy corn, and potato amylopectin (Hizukuri 1985).

Larger amounts of non-spherulitic material and less round spherulites were observed as the proportion of branched material increased, consistent with previous observations by Nordmark and Ziegler (2002a). Linear chains, such as amylose, would be expected to more easily fold to form the crystalline lamellae (Nordmark and Ziegler 2002a). The reduced extent of spherulitic crystallization caused by higher concentration of branches has also been reported for synthetic polymers (Chowdhury and others 1998; Jayakannan and Ramakrishnan 1999). In potato spherulites, as the ratio of amylose to amylopectin decreased, spherulites tended to agglomerate forming “bridges” among particles (Figure 3.7).

![Figure 3.7. SEM micrographs of spherulites made with various proportions of potato amylose and amylopectin. Scale bars =20 μm.](image)

Various microscopy techniques were used to observe the internal structure of starch spherulites. The characteristic internal radial organization could be observed (Figure 3.8). SEM images showed few broken spherulites with what appears to be a large central cavity (white arrows in Figure 3.9). Nordmark and Ziegler (2002b) also reported the presence of a central cavity in Hylon VII spherulites. However, this cavity was not observed in TEM images and AFM images of cross-sections of spherulites. It may be possible that the cross-sections were not from a region close to the center of the...
spherulites and for that reason the central cavity was not observed. To eliminate this possibility, cross sections from various depths of the resin blocks containing the spherulites were obtained and evaluated under the light microscope. The central cavity was not observed in these cross-sections. It is possible that the cavities shown in Figure 3.9 may be from isolated cases of spherulites that broke more easily during sample preparation.

Figure 3.8. SEM image of a broken spherulite made from 100% amylose from Hylon VII.

TEM images of intermediate material and amylopectin spherulites showed small holes away from the center, which were more numerous and larger in amylopectin spherulites (Figure 3.10). Images of cross-sections of spherulites showed that starch spherulites may develop from a sheaf-like precursor (shown with white arrows in Figure 3.10) similar to the spherulitic crystallization model for synthetic polymers (Figure 3.11) (Bassett and Keith 1984; Granasy 2006; Granasy and others 2005; Khoury and Passaglia 1976; Li and others 2000; Phillips 1994). In several images, a darker external region was observed and was more easily appreciated when the contrast of the image was increased (Figure 3.12). The average width of the darker outer region of spherulites made from Hylon VII amylose, intermediate material, and amylopectin was $2.7 \pm 0.8 \mu m$, $2.0 \pm 0.2 \mu m$, and $1.77 \pm 0.2 \mu m$, respectively ($n \geq 4$ spherulites, 10 measurement per spherulite).
Figure 3.9. SEM images of spherulites made from various proportions of Hylon VII amylose, intermediate material, and amylopectin. White arrows show what appears to be a central cavity.
Figure 3.10. TEM images of cross sections of spherulites made with various proportions of Hylon VII amylose, intermediate material, and amylopectin. White arrows show a sheaf-like structure.
Figure 3.11. Schematic representation of the development of a spherulite from small fibers through sheaving outlines to the final form. Adapted from Bassett and Keith (1984).
Figure 3.12. High contrast TEM images of cross sections of spherulites made with Hylon VII a) amylose, b) intermediate material, and c) amylopectin showing a darker outer region.
As explained in section 3.2.4, silver ions react preferentially with amorphous regions resulting in a darker appearance in TEM images. Therefore, the proportion of amorphous material towards the outside of the spherulites was higher than in the center. On the other hand, the lighter central region indicates a larger amount of crystalline material in the center of the spherulite. Figure 3.11 shows a schematic representation of the formation of a spherulite from a sheaf-like precursor, showing a central region with higher crystalline density.

Figure 3.13 shows AFM topographic images of cross-sections of spherulites made of amylose, amylopectin and intermediate material from Hylon VII. Topographic images (Figure 3.13) show a darker central region and a brighter outer area, which indicates that the outer region of the cross-sections of spherulites had taller features (see also Table 3.1). A similar observation was reported by Ridout and others (2004) for granules of wild-type pea starch. These authors explained that during sectioning, the interior of the granules is exposed, which can absorb water and swell during sample preparation. Then, when the excess water is removed, the central region collapses more than the outer region. To corroborate the effects of hydration on section swelling, Ridout and others (2004) encased starch granules by letting set a drop of a rapid-set Araldite-starch mixture on a mica surface. Then, the top surface was cut to produce a flat surface with starch granules with cut faces exposed. AFM images of the dried cut faces of encased granules were featureless, but after water exposure, images showed a large height variation between the outer and central region, which was not reversible after drying the wetted blocks for 3 months in a desiccator over P₂O₅. These authors hypothesized that the difference in height between the central and outer region was originated due to a difference in structure with different swelling and collapsing behavior. Similarly, it is possible that the structure of the central and the outer regions of the spherulites are different, resulting in a difference in swelling after sectioning of samples.
Figure 3.13. AFM topographic images of spherulites made from Hylon VII a) amylose, b) intermediate material, and c) amylopectin. Relative set point = 0.8. Scan size 10 µm x 10 µm.

Table 3.1. Roughness, blocklet size, wall thickness and height of starch spherulites made from different Hylon VII starch fractions (mean ± standard deviation)\(^1\).

<table>
<thead>
<tr>
<th>Material</th>
<th>RMS Roughness(^2,3)</th>
<th>Blocklet size(^2,3) (nm)</th>
<th>Outer region width(^2) (µm)</th>
<th>Outer region height(^2) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Outer region</td>
<td>Central region</td>
<td>Outer region</td>
<td>Central region</td>
</tr>
<tr>
<td>Amylose</td>
<td>3.9 ± 0.3aA</td>
<td>3.4 ± 0.1aB</td>
<td>20 ± 7aA</td>
<td>19 ± 7aA</td>
</tr>
<tr>
<td>Intermediate material</td>
<td>4.6 ± 0.1aA</td>
<td>3.2 ± 0.3aB</td>
<td>23 ± 8bA</td>
<td>25 ± 10bA</td>
</tr>
<tr>
<td>Amylopectin</td>
<td>4.7 ± 0.8aA</td>
<td>3.8 ± 1.5aA</td>
<td>26 ± 9bA</td>
<td>23 ± 8bA</td>
</tr>
</tbody>
</table>

\(^1\) RMS roughness and blocklet size were measured from 0.5 x 0.5 µm images
\(^2\) Same lower case letters within column indicate no significant differences at \(\alpha = 0.05\)
\(^3\) Same upper case letters within the same material indicate no significant differences (\(\alpha = 0.05\)) in roughness or blocklet size between the outer and central region.
Despite the difference in height between the center and the edges of the spherulites, both regions showed a granular structure (Figure 3.14–Figure 3.16) due to the presence of blocklets of approximately 19–26 nm (Table 3.1). In general, the outer region showed a rougher surface than the central region, which would be expected if the outer region had swollen giving rise to taller protuberances. No significant differences in roughness were observed among spherulites made of different starch fractions ($\alpha=0.05$) (Table 3.1). The blocklet structure could be identified in both topographic and phase images. The presence of 20–60 nm blocklets have also been reported in spherulites made from corn amylose with various DP (Creek 2007; Suwanayuen 2009). Blocklets of 20 nm to 500 nm in size have also been observed in starch granules from various botanical sources (Baker and others, 2001; Gallant and others, 1997; Ohtani and others, 2000; Ridout and others, 2003, 2004, 2006; Sujka & Jamroz, 2009; Szymonska & Krok, 2003). In starch granules, blocklets are believed to be formed by amylopectin side chains (Gallant and others 1997b), while in spherulites, blocklets can be formed by amylose (Creek 2007).

The granular appearance has also been observed in spherulites made of synthetic polymers. Ivanov and Magonov (2003) proposed that the granular structure of low density polyethylene (LDPE) spherulites observed in topographic images was caused by amorphous regions of polymer chains on the surfaces of the crystalline lamellae as depicted in Figure 3.17a. By increasing the tip-sample force, these authors showed a fibril structure instead of a granular appearance suggesting that fibrils and lamellae were formed by a crystalline core. However, in the present investigation, the granular appearance persisted when the tip-sample force was doubled (Figure 3.18). Higher sample tip force could cause damage to the surface of the sample or the tip itself. It could be possible that the tip sample force was not increased enough to go through the amorphous patches, and therefore, the granular appearance remained. However, phase images contradicted the idea that the granular appearance is caused by the amorphous patches described by Ivanov and Magonov (2003).
Figure 3.14. AFM topographic (left) and phase (right) images of the outer (a and b) and central (c and d) regions of Hylon VII amylose spherulites. Relative set point = 0.8. Scan size 0.5 µm x 0.5 µm.
Figure 3.15. AFM topographic (left) and phase (right) images of the outer (a and b) and central (c and d) regions of Hylon VII intermediate material spherulites. Relative set point = 0.8. Scan size 0.5 µm x 0.5 µm.
Figure 3.16. AFM topographic (left) and phase (right) images of the outer (a and b) and central (c and d) regions of Hylon VII amyllopectin spherulites. Relative set point = 0.8. Scan size 0.5 µm x 0.5 µm.
Phase images of spherulites (Figure 3.14–Figure 3.16, and Figure 3.18) also show a granular appearance. The lighter color represents harder regions and darker color softer regions. The brighter color of the blocklets surrounded by darker regions suggests that blocklets were composed of more crystalline regions surrounded by amorphous material. It is also possible that during sample preparation, the resin or the agar infiltrated inside the spherulites, and the observed granular structure represents crystalline or semi-crystalline blocklets surrounded by the infiltrated resin or agar. Strobl (2006) hypothesized that during crystallization of synthetic polymers, a transient intermediate ‘granular’ stage occurs as the crystalline lamellae is developed (Figure 3.17b). The crystalline units from the ‘granular’ stage will merge to form the complete crystalline lamellae given sufficient time for their formation (Strobl 2000). However, the intermediate ‘granular’ stage may be preserved if the mobility of the crystalline segments is limited, for example by quickly cooling the polymer below Tg or by the structure of the crystalline units, such as a stable packing of helices forming the crystalline units (Creek 2007).
Figure 3.18. AFM topographic (left) and phase (right) images of the outer region of a Hylon VII amylose spherulite obtained using light tapping (a and b) and hard tapping (c and d) mode. Light tapping mode relative set point = 0.8 and hard tapping mode relative set point = 0.4. Scan size 0.5 µm x 0.5 µm.

If we consider that the central region of the spherulite is the region where the spherulite was originated, then the crystalline units initially formed in the central area may have started coming into proximity to form the crystal lamella. Then, less amorphous material may be surrounding the blocklets, and therefore swelling may be limited. On the other hand, in the outer region, crystal units may be surrounded by more amorphous material that can absorb water and swell during sample preparation giving rise to the observed taller features of the outer region of spherulites. The idea of larger
proportions of amorphous material in the outer region is in agreement with the darker outer regions observed in TEM images (Figure 3.12).

No significant differences ($\alpha=0.05$) were observed between the size of the blocklets of the central and outer regions of spherulites made of the same starch fraction (Table 3.1). However, blocklet size varied depending on the starch fraction. Smaller blocklets were observed in amylose spherulites, and no significant size differences ($\alpha=0.05$) were observed between the blocklets of amylopectin and intermediate material (Table 3.1). In granular starches, blocklets are believed to be formed by a group of clusters of amylopectin side chains (Gallant and others 1997b). These clusters have an average diameter of 9–10 nm (Gallant and others 1997b; Jenkins and others 1993; Jenkins and Donald 1995) and are composed by a crystalline region of double helices and an amorphous region containing the branch points (Gallant and others 1997b). The average cluster size is independent of the botanical source (Jenkins and others 1993; Jenkins and Donald 1995), but the size of the crystalline region within a cluster varies among starches. For example, amylomaize starch clusters have a larger crystalline region as compared to those from normal maize starch (Jenkins and Donald 1995). If blocklets of amylopectin spherulites (20 nm approximately) are also formed by a group of these clusters like in granular starches, then amylopectin spherulite blocklets may be formed by two clusters of amylopectin side chains.

The extent and height of the outer region with taller features varied depending on the starch fraction. Amylose spherulites had a wider region with taller features compared to amylopectin and intermediate material spherulites (Table 3.1). It is possible that the branch structure of amylopectin holding the clusters together, limited the absorption of water and swelling during sectioning of samples. Klucinec and Thompson (1998) reported that the structure of the intermediate material of Hylon VII starch is more similar to amylopectin than to amylose, and therefore, a similar swelling behavior of spherulites made of intermediate material and amylopectin is not surprising.
A section of Hylon VII amylose spherulites was treated with HCl 2N for 1 h to remove the amorphous and less crystalline part of sectioned spherulites. AFM images of the acid treated spherulites (Figure 3.19) showed larger blocklets than their non-acid treated counterpart (Figure 3.14). Some of these larger blocklets appeared to be formed by a stack of layers (see white ovals in Figure 3.19b) with an average thickness of 17.6 ± 5.04 nm. Coincidently, this thickness was not significantly different (p>0.05) than the blocklet size of amylose spherulites before acid treatment (Table 3.1). It is possible that these layers represent crystalline lamella that stack together forming larger blocklets.

**Thermal characterization**

An endothermic transition was observed in all samples made of Hylon VII fractions and can be attributed to the dissolution of spherulites. The dissolution temperature increased with amylose concentration and decreased with amylopectin content (Figure 3.20b). Chowdhury and others (1998) also reported that the melting temperature of LDPE spherulites decreased with higher concentration of branch points. The longer amylose chains, as compared to intermediate material and amylopectin chains, may allow the formation of longer helices with a more heat stable conformation and thus, a higher dissolution temperature. The shorter chains of amylopectin as compared to those of intermediate material (Klucinec and Thompson 1998) resulted in the formation of shorter helices with lower thermal stability and thus, a lower dissolution temperature.
Figure 3.19. AFM topographic (top) and phase (bottom) images of the outer region of a Hylon VII amylose spherulite treated with 2N HCl for 1 h. Relative set point = 0.8. Scan size 1 µm x 1 µm. White ovals show the formation of layered structures.
Figure 3.20. Thermal characterization of starch spherulites made with various proportions of amylose, amylopectin, and intermediate material from Hylon HVII: (a) dissolution onset temperature, (b) dissolution peak temperature, (c) dissolution temperature range, and (d) dissolution enthalpy.
The range of dissolution temperature was also affected by the proportion and type of material (Figure 3.20c and Table 3.2). A larger dissolution range indicates a higher polydispersity of lamella sizes. The shortest dissociation temperature range was observed for amylopectin spherulites. If the cluster model of the amylopectin molecule is considered, then the length and packing of double helices would probably be more uniform. The largest dissociation temperature range was observed for samples made of intermediate material, and samples made of amylose/amylopectin mixtures. It would be expected that the large differences between amylose and amylopectin molecules may result in large diversity of helical sizes. The structure of the intermediate material is not well understood. Baba and Ari (1984) suggested that the intermediate material consists in four or five branches of around 50 DP linked to a main 100–150 DP linear chain. Klucinec and Thompson (1998) suggested, based on the similar chain length distribution, that the gross branching structure is similar to that of amylopectin but with some structural feature differences. It is possible that the structure of the intermediate material does not follow the cluster model and a wide variety of helical lengths are formed resulting in a wide range of dissolution temperature.

The dissolution enthalpy increased with amylose content suggesting a higher degree of crystallinity of samples made with higher proportions of amylose. This observation is consistent with the more spherulites and less amount of non-spherulitic material observed at higher amylose concentrations. However, in samples made with various proportions of amylopectin and intermediate material, the dissolution enthalpy increased at higher amylopectin concentrations, even though fewer spherulites were observed. For these samples, the onset temperature of the observed endotherms was between 71 °C and 74 °C (Figure 3.20a). It is possible that the endotherm does not only represent the dissolution of spherulites, but also, the dissolution of retrograded non-spherulitic amylopectin.
Table 3.2. Regression coefficients for the effect of Hylon VII amylose, amylopectin, and intermediate material on thermal properties of spherulites. Star (*) shows significant regression coefficients at $\alpha=0.05$.

<table>
<thead>
<tr>
<th></th>
<th>Onset dissolution temperature</th>
<th>Peak dissolution temperature</th>
<th>Dissolution temperature range</th>
<th>Dissolution enthalpy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylose</td>
<td>88*</td>
<td>118*</td>
<td>57*</td>
<td>22*</td>
</tr>
<tr>
<td>Intermediate material</td>
<td>73*</td>
<td>96*</td>
<td>64*</td>
<td>4</td>
</tr>
<tr>
<td>Amylopectin</td>
<td>73*</td>
<td>93*</td>
<td>53*</td>
<td>12*</td>
</tr>
<tr>
<td>Amylose*Intermediate material</td>
<td>2</td>
<td>23</td>
<td>2</td>
<td>-7</td>
</tr>
<tr>
<td>Amylose*Amylopectin</td>
<td>-21</td>
<td>-19</td>
<td>41</td>
<td>-17</td>
</tr>
<tr>
<td>Intermediate material*Amylopectin</td>
<td>16</td>
<td>8</td>
<td>-21</td>
<td>8</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.81</td>
<td>0.91</td>
<td>0.68</td>
<td>0.90</td>
</tr>
</tbody>
</table>
Spherulites made from common corn and potato starch fractions showed a dissolution endotherm at around 123–124 °C, and around 110°C, respectively (Figure 3.21 and Figure 3.22). The dissolution peak temperature of these spherulites did not decrease with higher proportions of amylopectin, suggesting that in common corn and potato starch spherulites, amylopectin does not co-crystallize with amylose to form the spherulites. Similar to spherulites from Hylon VII fractions, the enthalpy of spherulite dissolution decreased as the ratio of amylose to amylopectin decreased (Figure 3.21 and Figure 3.22). This observation is consistent with the fewer spherulites observed at lower amylose concentrations. In samples containing potato amylose, a second endotherm was observed at around 155 °C, possibly representing the melting of retrograded amylose (Figure 3.22). The small endotherm observed in 100% common corn or potato amylopectin samples may be due to the dissolution of small crystallites like the small birefringent particles observed in Figure 3.6.

3.4. Conclusions

Spherulitic crystallization of starch fractions and their mixtures is greatly affected by the proportion and structure of each starch fractions. Higher ratios of linear to branched molecules resulted in the formation of larger amounts of rounder spherulites with higher thermal stability. In addition to the presence of branches, it appears that spherulitic crystallization is also affected by other factors, such as degree of branching, chain length, and chain length distribution. Starch spherulites may develop from a sheaf-like precursor, and have an internal radial organization and blocklet structure similar to some synthetic spherulites. Because the ratio of amylose to amylopectin and the molecular structure of the starch fractions can affect the spherulitic crystallization of starch, these factors should be taken into account when selecting a starch source for spherulite formation.
Figure 3.21. Dissolution endotherm of spherulites made with various proportions of common corn amylose and amylopectin. %amylose/%amylopectin from bottom to top: 100/0, 67.7/33.3, 33.3/67.7, and 0/100.
Figure 3.22. Dissolution endotherm of spherulites made with various proportions of potato amylose and amylopectin. %amylose/%amylopectin from bottom to top: 100/0, 80/20, 60/40, 40/60, 20/80, and 0/100.

3.5. References


Fanta GF, Felker FC, Shogren RL, Salch JH. 2006. Effect of fatty acid structure on the morphology of spherulites formed from jet cooked mixtures of fatty acids and defatted corn starch. Carbohydrate Polymers 66(1):60-70.


Chapter 4

FORMATION OF INCLUSION COMPLEXES OF STARCH WITH FATTY ACID ESTERS OF BIOACTIVE COMPOUNDS

4.1. Introduction

The ability of nutraceuticals to provide health benefits make these compounds attractive to consumers (Cohen and others 2008). Because of these apparent health benefits, interest in bioactive compounds in foods is growing (Shimoni 2009) resulting in an increasing demand for more nutritive functional foods. However, most phytonutrients found in fruits and vegetables are bitter, acrid or astringent, which makes the use of these bioactive compounds a challenge to functional food designers (Drewnowski and Gomez-Carneros 2000). Many bioactive compounds are also sensitive to thermal and oxidative stress and can be easily degraded during processing or storage, and possibly during their passage through the gastrointestinal tract after consumption. In addition, their solubility in water is often limited and, therefore, a vehicle to increase their dispersability in a hydrophilic food environment is necessary. Encapsulation can provide to these compounds increased stability during processing and storage, and may protect the bioactive components during transit through the gastrointestinal tract while providing controlled release properties. Protecting fragile bioactives during transit through the acidic environment of the stomach may insure their release in the small intestine where they are absorbed into the blood stream (Shimoni 2009). In addition, this type of controlled release capsule may prevent the perception of undesirable sensory attributes that are associated with some bioactive compounds.

Fortification of food products with vitamins is a common practice in the food industry. Thus, methods to improve stability of vitamins and their controlled release are
of great interest. The relationship between fruit and vegetable consumption and the reduced rates of cancer and coronary heart disease has been widely recognized. For example, phytosterols, found in fruits and vegetables, are well known to reduce low density lipoprotein-cholesterol levels lowering the risk of cardiovascular diseases (Moreau and Hicks 2004).

The use of starch in the pharmaceutical industry has grown in recent years, for example, to produce particles with a biodegradable matrix for subcutaneous or nasal delivery (Eliasson and Wahlgren 2004). Amylose (McConnell and others 2007; Milojevic and others 1996a, 1996b) and high amylose starch (Freire and others 2009b; Podczeck and Freire 2008) have also been studied for colon specific drug delivery in oral formulations, where the controlled release mechanism is the enzymatic degradation of the starch in the colon (Eliasson and Wahlgren 2004; Freire and others 2009a; McConnell and others 2007; Milojevic and others 1996a, 1996b). Biodegradability, low toxicity, food grade, and availability are some of the benefits in using starch for encapsulation purposes. In addition, its physicochemical properties, including retrogradation (recrystallization), film forming ability, complex formation, and resistance to various degrees towards amylase hydrolysis, make starch a promising material for delivery systems.

Amylose and amylopectins are well known to form inclusion complexes with some low molecular weight polar and non-polar substances such as iodine, alcohols, fatty acids, aromas (Rondeau-Mouro and others 2004), DMSO (Godet and others 1995b), salicylic acid and its analogues (Oguchi 1998; Uchino and others 2002), p-aminobenzoic acid (Tozuka and others 2006), ibuprofen, warfarin (Hong and others 1998), and organic dyes (Kim and others 1996). When these guest molecules are present, amylose forms a left-handed single helix stabilized by hydrogen bonds (Conde-Petit and others 2006), with a hydrophilic outside surface and a hydrophobic inside helical channel (Immel and Lichtenthaler 2000). These amylose single helices can arrange forming a crystalline structure known as V-type (Biais and others 2006). Guest molecules are usually included
within the single helix, but can also be trapped between helices (Rondeau-Mouro and others 2004).

Amylose-lipid complexes form two types of polymorphic structures described as low-melting form, also called type I or form I, and high-melting form, also called type II or form II (Biliaderis 1992; Biliaderis and Galloway 1989; Eliasson 1994). Type I inclusion complexes have little crystallographic register (amorphous X-ray pattern), and are assumed to be formed by helical segments randomly distributed (Biliaderis 1992; Biliaderis and Galloway 1989), although a diffused V-type pattern can be observed after freeze-drying and rehydrating type I complexes (Seneviratne and Biliaderis 1991). On the other hand, type II complexes are polycrystalline aggregates (Biliaderis 1992) and display the characteristic V-type X-ray diffraction pattern, have birefringent properties, and a more compact organization than type I complexes. The complexation temperature generally used to form type I and type II complexes is 60 °C and 90 °C, respectively (Gelders and others 2005).

Even though the formation of inclusion complexes of a wide variety of small molecules and amylose has been known for many years, only recently, have these amylose inclusion complexes been seriously investigated as a delivery system of non-drug bioactive compounds (Cohen and others 2008; Lalush and others 2005; Lesmes and others 2008; Lesmes and others 2009; Zabar and others 2009), including conjugated linoleic acid (Lalush and others 2005; Yang and others 2009) and genistein (Cohen and others 2008). These investigations suggested that inclusion complexes with bioactives can protect these molecules during the passage through the stomach, and be released in the small intestine by the action of enzymes on the amylose complexes (Lalush and others 2005; Yang and others 2009).

The formation of amylose-inclusion complexes depends on the size, shape, and solubility of the guest molecules. If guest molecules are too water soluble, for example ascorbic acid, then they will not effectively partition into the starch matrix. In other
cases, if the molecules are too large, for example retinol or phytosterols, then they will not fit within the helical cavity that amylose forms when complexing with the ligands. Given the well known ability of amylose to form inclusion complexes with fatty acids, the use of fatty acid esters of these compounds may be an interesting approach for the formation of inclusion complexes with these bioactive molecules.

Encapsulation of bioactive compounds using a commercial native starch would be of relevance because of the lower cost as compared to prepared amylose (Tapanapunnitikul and others 2008). However, the presence of lipids present in native starch can interfere with starch complexation with certain compounds, for example with iodine (Hizukuri and others 2006). In other cases, the presence of native lipids can favor complex formation, such is the case with low-solubility flavors (Tapanapunnitikul and others 2008).

The potential use of starch inclusion complexes as delivery systems for drugs or other bioactive compounds may be promising. However, increased knowledge and better understanding of the formation of starch inclusion complexes with various molecules is essential (Eliasson and Wahlgren 2004). The objectives of the present study were to: a) investigate the formation of amylose and high amylose starch inclusion complexes with ascorbyl palmitate, retinyl palmitate, and phytosterol esters; b) evaluate the effect of native lipids on complex formation of high amylose starch inclusion complexes with ascorbyl palmitate, retinyl palmitate, and phytosterol esters; and c) evaluate the effect of addition of various concentrations of fatty acid esters on the formation of high amylose starch inclusion complexes with ascorbyl palmitate, retinyl palmitate, and phytosterol esters.
4.2. Materials and methods

4.2.1. Materials

High amylose maize starch (Hylon VII) was supplied by National Starch and Chemical Company (Bridgewater, NJ, USA). Dimethyl sulfoxide (DMSO) and \( d_6 \)-DMSO were obtained from Sigma-Aldrich, Inc (St. Louis, MO, USA). The evaluated fatty acid esters were ascorbyl palmitate, retinyl palmitate, and phytosterol esters (Figure 4.1). Ascorbyl palmitate and retinyl palmitate were acquired from Sigma-Aldrich, Inc (St. Louis, MO, USA). Phytosterol esters were CoroWise\textsuperscript{TM} plant sterol esters donated by Cargill Incorporated (Wayzata, MN, USA). Based on the manufacturer, CoroWise\textsuperscript{TM} plant sterol esters are esters of various phytosterols (40–58% sitosterol, 20–28% campesterol, and 14–23% stigmasterol) with canola oil fatty acids. Canola oil fatty acids are composed mainly of unsaturated fatty acids, approximately 62% of oleic (C18:1), 21% of linoleic (C18:2), and 8% of linolenic acid (C18:3) (Warner and others 1994).

4.2.2. Sample preparation

Defatted Hylon VII starch and non-granular waxy maize starch were produced by dispersing 10 g of starch in 200 mL of a 90% DMSO aqueous solution in a boiling water bath with constant stirring for 3 h. The mixture was divided in four 250 mL centrifuge bottles. Starch was precipitated by adding 4 volumes of ethanol and recovered by centrifugation at 6500 \( g \) for 15 min at 4 \( ^\circ \)C. Pellets were washed with ethanol (75 mL/bottle) and centrifuged as described above. The washing procedure was repeated one more time with ethanol and one time with acetone. The precipitate was dried in an oven at 50 \( ^\circ \)C for 24 h and stored in a desiccator until used (Klucinec and Thompson 1998). Amylose and amylopectin fractions of Hylon VII were obtained by differential alcohol precipitation as described by Klucinec and Thompson (1998) and in Section 3.2.2.
Starch inclusion complexes were prepared by the DMSO method as described by Godet and others (1993) with slight modifications. A sample of 0.5 g of amylose, amylopectin, native Hylon VII, or defatted Hylon VII was dispersed in 10 mL of a 95% DMSO aqueous solution in a boiling water bath with constant stirring for at least 1 h. Then, 1 mL of a fatty acid ester solution (1:10 guest molecule:starch) in 95% DMSO was prepared and mixed with the starch solution at 90 °C. The sample was vortexed, and kept at 90 °C for 1 h in a water bath. After this time, 25 mL of water at 90 °C were added to the sample tube, and mixed using a vortex. The water bath was turned off and samples were allowed to slowly cool inside the water bath for 24 h. The final temperature of the
system after cooling was approximately 28 °C. Inclusion complexes were recovered by centrifugation at 3700 rpm for 15 min, washed three times with 40 mL of a 50/50 ethanol/water solution, and recovered by centrifugation (Godet and others 1995). The final precipitates were washed with 100% ethanol, filtered, rinsed with excess ethanol, and dried at room temperature in a desiccator. Dried samples were weighed and stored in the freezer (-15 °C) until used. Yield of the precipitates were calculated as grams of precipitate divided by the total amount of starch and fatty acid ester used, multiplied by 100.

In order to evaluate the effect of the amount of fatty acid ester added during processing on complex formation, native Hylon VII starch inclusion complexes were also prepared using various amounts of fatty acid esters (2.5% to 50% w/w Hylon VII).

4.2.3. Wide angle X-ray Diffraction

Wide angle X-ray diffraction patterns were obtained with a Rigaku MiniFlex II desktop X-Ray diffractometer (Rigaku Americas Corporation, TX, USA). Samples were equilibrated for 48 hours over a supersaturated aqueous solution of KCl at room temperature (~85% relative humidity). After equilibration, samples were lightly pressed into a 10 mm disk using an Econo-press die (Spectra Tech Econo-press kit, Thermo Scientific), placed on to a glass holder, and exposed to a Cu Kα radiation (λ=0.15 nm). A current of 15 mA and 30 kV were used. Samples were continuously scanned between 4° and 30° at a scan speed of 1°/min with a step size of 0.02°. The X-ray diffraction data was analyzed with Jade v.8 software (Material Data Inc., Livermore, CA, USA).

To calculate the percentage of crystallinity (% crystallinity) in a sample, an amorphous halo was subtracted from the overall X-ray diffraction pattern. The overall area was calculated as the area between the linear baseline and data points (Figure 4.2). The amorphous halo was generated by Jade software using the cubic spline fit option. The generated amorphous halos were asymmetric similar to the asymmetric amorphous
pattern of non-granular waxy maize starch (Figure 4.3). For samples with an X-ray diffraction pattern displaying distinct peaks, the amorphous halo was generated by fitting the cubic spline starting at 4°, and continuing to 5°, 6°, 10°, 16°, 18°, 23.5°, 27°, and 30° diffraction angles along the diffraction pattern. With this method, the generated amorphous halo generally connected the base of the peaks (Figure 4.2a). Hylon VII-ascorbyl palmitate diffractograms displayed broad peaks (Figure 4.2b) similar to those reported by Evans (2005) for high amylose starch precipitated with ethanol from NaOH solutions. The amorphous halo for Hylon VII-ascorbyl palmitate precipitates was generated by fitting a cubic spline starting at 4°, and continuing to 5°, 10°, 16°, 25°, 28°, and 30° diffraction angles along the diffraction pattern (Figure 4.2b). The % crystallinity calculated by this method was in agreement with the range of % crystallinity calculated using the two methods proposed by Evans (2005) for this type of X-ray diffraction patterns. The % crystallinity was calculated as the proportion of the crystalline area to the overall area multiplied by 100 (Creek 2007; Evans 2005; Rindlav and others 1997).

The relative crystal size of inclusion complexes was estimated from the broadening of the main peak reflections at 2θ around 13° and 20° by Jade software after peak decomposition, using the Scherrer’s equation (Eq. 1):

\[
D_{(hkl)} = \frac{0.9 \times \lambda}{\beta_{1/2} \times \cos \theta}
\]  

(Eq. 1)

where \(D_{(hkl)}\) is the mean crystal size, \(\lambda\) represents the wave length of the X-ray used (0.15 nm), \(\beta_{1/2}\) is the full width at half maximum, and \(\theta\) is the Bragg angle (Hizukuri and Nikuni 1957). Peak reflections at 2θ ≈ 13° and 2θ ≈ 20° correspond to the planes (130) and (310) of the \(V_{61}\) orthorhombic unit cell, respectively (Le Bail and others 2005; Rondeau-Mouro and others 2004; Yamashita 1965). Jade software estimates the average crystal size after correcting \(\beta_{1/2}\) for instrumental peak broadening.
Figure 4.2. Illustration of the baseline and amorphous halo to calculate the % crystallinity of a) samples showing distinct peaks, and b) Hylon VII-ascorbyl palmitate samples showing broad peaks.
Figure 4.3. X-ray diffractogram of non-granular waxy maize starch showing an asymmetric amorphous pattern.

4.2.4. Thermal analysis

Approximately 5 mg of sample were weighed in a 60 µL stainless steel differential scanning calorimeter (DSC) pan (Perkin-Elmer Instruments, Norwalk, CT, USA) and water was added to obtain a 10% dispersion (w/w). The pans were hermetically sealed and stored overnight for moisture equilibration. Samples were analyzed using a Thermal Advantage Q100 DSC (TA Instruments, New Castle, DE, USA). Initially, samples were equilibrated to 10°C, and then heated to 180 °C at 10 °C/min. The DSC was calibrated with indium, and an empty sample pan was used as a reference. The baseline was obtained by processing two empty pans using the same heating treatment and subtracted from the data. Data was analyzed using the TA Universal Analysis software (Universal Analysis 2000 v.4.2E, TA Instruments-Waters LLC, New Castle, DE, USA).
4.2.5. Quantification of complexed fatty acid esters by Fourier transformed infrared (FTIR) spectroscopy

The amount of fatty acid esters in the complexes was estimated using a Bruker IFS 66/S FTIR Spectrometer (Bruker Optics, Billerica, MA, USA). Approximately 3–5 mg of sample were rigorously mixed with a pre-weighed amount of KBr for 30 s using a Wig-L-Bug amalgamator. A sample of 70 mg of this mixture was pressed into a pellet using a Quick Press (International Crystal Laboratories). Spectra were obtained in transmission mode from 500 cm$^{-1}$ to 4000 cm$^{-1}$ at a resolution of 6 cm$^{-1}$.

Quantity of entrapped molecules in starch samples was calculated by subtracting the Hylon VII starch FTIR spectra from the inclusion complexes spectra after water vapor correction. After subtraction, the area under the peak corresponding to the carbonyl group was calculated and normalized to 1 mg of sample. For retinyl palmitate and phytosterol ester, the carbonyl peak was around 1738 cm$^{-1}$ and 1737 cm$^{-1}$, respectively. In the case of ascorbyl palmitate, two overlapping carbonyl peaks were identified between 1715 cm$^{-1}$ and 1800 cm$^{-1}$ and the combined area under the two peaks was used for quantification.

Fanta and Salch (1991) reported that the FTIR spectrum of corn starch was affected by sample particle size. Particles larger than the IR radiation wavelength will cause light scattering affecting absorption values. However, carbonyl absorbance was less sensitive to sample preparation conditions. In order to verify that particle size did not significantly affect carbonyl absorbance, samples of complexes were ground in a steel vial containing a steel ball pestle. Before grinding, the vial was submerged in liquid nitrogen to avoid breakdown of the fatty acid ester due to the increased temperature caused by grinding. Samples were pulverized for 30 s using a Wig-L-Bug amalgamator. No significant differences (t-test, p>0.05) were observed between the amount of fatty acid ester of the original and pulverized samples.
To show that ethanol washing was sufficient to remove the free retinyl palmitate and phytosterol esters from the precipitates, samples were washed with hexane and analyzed using the FTIR. Approximately 10 mg of precipitate were washed with 100 µL hexane for 20 min. Samples were manually shaken every 5 min. Precipitates were collected by centrifugation, washed with 200 µL hexane, and collected again by centrifugation. Samples were placed in a desiccator to allow the remaining hexane to evaporate. Then, the amount of fatty acid ester in the precipitates after hexane washing was estimated using the FTIR as described above. Analysis of variance was used to evaluate significant differences in the amount of fatty acid esters before or after washing the precipitates with hexane.

Calibration standard curves were generated using physical mixtures of the fatty acid esters with native Hylon VII at concentrations from 0.5% to 10% (w fatty acid ester/w dry starch). To prepare standard mixtures, fatty acid esters were first dispersed in ethanol, then Hylon VII was mixed into this dispersion, and finally, ethanol was evaporated.

4.2.6. Nuclear magnetic resonance (NMR)

Ascorbyl palmitate-Hylon VII inclusion complexes were characterized using $^1$H NMR as described by Wulff and others (2005) with slight modifications. Approximately 25 mg of sample were dissolved in 0.7 mL of d$_6$-DMSO at 60 °C in a water bath for 40 min mixing every 8 min using a vortex. $^1$H spectra of samples dissolved in d$_6$-DMSO were obtained at 400.13 MHz on a Bruker DRX-400 NMR spectrometer operating in the quadrature mode at 25 °C using 30 degree pulses and 8.95 seconds between scans to guarantee complete relaxation and quantitative integration. Spectra were referenced to the residual $^1$H signal of d$_6$-dimethylsulfoxide ($\delta = 2.49$ ppm). The percentage of ascorbyl palmitate in the sample was calculated from the integration of the proton signal corresponding to the CH$_3$ from the end of the alkyl chain between 0.80 ppm and 0.88
ppm in relation to the integration of the proton signal of carbon 1 of the anhydroglucose units (AGU) between 4.95 ppm and 5.20 ppm (Figure 4.4).

Figure 4.4. $^1$H NMR representative spectrum of Hylon VII-ascorbyl palmitate precipitates dissolved in d$_6$-DMSO.

4.3. Results and Discussion

4.3.1. Inclusion complexes with amylose and amylopectin

The formation of amylose inclusion complexes with ascorbyl palmitate, retinyl palmitate and phytosterol esters was evaluated. The three fatty acid esters induced amylose precipitation, however only retinyl palmitate induced amylopectin precipitation. Starch can precipitate out of solution by simple retrogradation or complex formation (Tapanapunmitikul and others 2008). X-ray diffraction patterns of the obtained amylose precipitates with the three guest molecules showed a V-type diffraction pattern indicating
the formation of inclusion complexes between amylose and these fatty acid esters (Figure 4.5). Complexation was carried out at 90 °C which has been shown to generally favor the formation of type II complexes (Gelders and others 2005; Karkalas and others 1995). A V-type diffraction pattern is usually associated with type II inclusion complexes (Biliaderis 1992; Biliaderis and Galloway 1989). However, Seneviratne and Biliaderis (1991) reported that freeze-dried and rehydrated type I inclusion complexes can display a diffused V-type pattern. In the present investigation, complexes were dried and equilibrated at 85% relative humidity, so the possibility of these complexes to be type I cannot be eliminated.

![Figure 4.5. X-ray diffraction patterns of a) amylose-ascorbyl palmitate, b) amylose-retinyl palmitate, c) amylose-phytosterol ester, and d) amylopectin-retinyl palmitate precipitates.](image)

The highest yields of precipitate were observed for samples made with ascorbyl palmitate, followed by retinyl palmitate, and then by phytosterol ester (Table 4.1). The alkyl chain of fatty acid esters is presumed to be included inside the helical cavity similar to the currently accepted model for amylose-fatty acid inclusion complexes (Godet and others 1995b). Godet and others (1995b) prepared amylose-palmitic acid complexes
using a similar method used in this study, and reported a yield of 96.6% complexed amylose (DP 900) when 10% palmitic acid (w/w amylose) was used. In the present investigation, only 61.7% yield was obtained when using 10% ascorbyl palmitate (w/w amylose). The weight fraction of palmitic acid in ascorbyl palmitate is around 0.62. Thus, the lower yield of amylose-ascorbyl palmitate precipitates is likely due to the lower proportion of added palmitic acid. The yield of amylose-retinyl palmitate complexes was 11.2% and the yield of amylose-phytosterol ester complexes was 6.5%. The weight fraction of fatty acids in retinyl palmitate and phystosterol ester is 0.49 and 0.40, respectively. Thus, the low yield of amylose complexes with these two compounds was not only caused by the lower proportion of added fatty acid.

Tufvesson and others (2003b) explained that a balance between fatty acid availability and complex formation rate determine the amount of complexes formed. Tapanapunnitikul and others (2008) reported little yield of precipitate when low solubility compounds were used. These authors proposed that a lower solubility of guest molecules in the reaction medium results in less available molecules for complexation, and shorter and less stable complexes. Individual complexes with lower stability will be less likely to induce nucleation and further crystallization, resulting in lower precipitation. Ascorbyl palmitate is an ester of palmitic acid with a hydrophilic ascorbic acid molecule. On the other hand, retinyl palmitate and phystosterol esters are esters of fatty acids with hydrophobic molecules. Ascorbic acid is highly soluble in water (\( \sim 3.3 \times 10^4 \text{ mg ascorbic acid/100 mL water} \)) (Budavari and others 1989), while retinol (\( \sim 1.7–1.8 \text{ mg retinol/100 mL water} \)) (Szuts and Harosi 1991) and sterols (0.2 mg cholesterol/100 mL water) (Budavari and others 1989) are practically insoluble in water. Esterification of these molecules with long chain fatty acids decreases their water solubility (Drabent and others 1997; Law 2000). For example, the solubility of ascorbyl palmitate (\( 5.6 \times 10^2 \text{ mg ascorbyl palmitate/100 mL water} \)) is much lower than that of ascorbic acid (Swern 1949). Thus, the higher solubility of ascorbyl palmitate as compared to the other ligands could be one of the major reasons of the much higher yield of amylose-ascorbyl palmitate precipitates.
Table 4.1. Yield, % crystallinity, % entrapped fatty acid ester, and stoichiometry of amylose and amylopectin precipitates with ascorbyl palmitate, retinyl palmitate, or phytosterol esters.

<table>
<thead>
<tr>
<th>Fatty acid ester</th>
<th>Yield of precipitate (g precipitate/100 g starch+fatty acid ester)</th>
<th>% Crystallinity</th>
<th>% fatty acid ester in precipitate (w/w dry starch)</th>
<th>Mols AGU/mol complexed fatty acid ester</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amylose</td>
<td>Amylopectin</td>
<td>Amylose</td>
<td>Amylopectin</td>
</tr>
<tr>
<td>Ascorbyl palmitate</td>
<td>61.7 ± 0.3a</td>
<td>No precipitate</td>
<td>35 ± 3 a</td>
<td>NA</td>
</tr>
<tr>
<td>Retinyl palmitate</td>
<td>11.2 ± 0.8 b</td>
<td>2.3</td>
<td>23 ± 2 b</td>
<td>16 ± 8</td>
</tr>
<tr>
<td>Phytosterol ester</td>
<td>6.5 ± 0.4 c</td>
<td>No precipitate</td>
<td>29 ± 1 ab</td>
<td>NA</td>
</tr>
</tbody>
</table>

1. Within column, mean ± standard deviation followed by different letter are significantly different at α=0.05
2. Reported values of % ascorbyl palmitate in precipitate are the adjusted FTIR values.
3. Stoichiometry of phytosterol ester complexes was calculated based on sitosterol oleate molecular weight.
NA = Not available because no precipitate was obtained.
AGU = Anhydro glucose units.
The % crystallinity was 35%, 23%, and 29% for amylose-ascorbyl palmitate, -retinyl palmitate, and -phytosterol ester complexes, respectively (Table 4.1). The observed percentages of crystallinity were within the range previously reported for V-type starch (Evans 2005). The % crystallinity depends on the ability of individual complexed amylose helices to pack into a crystallite. The difference in the ability of single helices to arrange into an ordered structure could be the result of the structure of the molecule forming the ester with the fatty acid (i.e. ascorbic acid, retinol, or phystosterols). The small ascorbic acid molecule is less likely to significantly disturb the packing of single helices resulting in a higher % crystallinity. On the other hand, the larger and more flexible retinol molecule is more likely to interfere in the packing of single helices. In addition, retinol may get more entangled with amylose chains, possibly increasing the proportion of amorphous regions.

Thermograms of amylose-ascorbyl palmitate precipitates showed a single endotherm around 100 °C (Figure 4.6a). This endotherm is attributed to the dissociation of inclusion complexes through a helix → coil transition (Biliaderis and Seneviratne 1990; Karkalas and others 1995; Raphaelides and Karkalas 1988; Seneviratne and Biliaderis 1991). The transition temperature of type II amylose-palmitic acid complexes has been reported to be around 118 °C (Tufvesson and others 2003b). Thus, if amylose-ascorbyl palmitate complexes are type II, then these complexes had a lower thermal stability compared to its palmitic acid counterpart.

Eliasson (1994) explained that the polar head of the lipid can affect the thermal transition of amylose-lipid complexes. For example, Tufvesson and others (2003a, 2003b) showed that amylose-monoglyceride complexes have a lower dissociation temperature as compared to their amylose-fatty acid counterparts. These authors reported 79.6 °C and 90.3 °C as the two transition temperatures observed for amylose-glycerol monopalmitin complexes. Similarly, the polar head of ascorbyl palmitate affected the thermal stability of helical complex between amylose and the aliphatic chain. It is
possible that ascorbyl palmitate forms shorter helices of amylose inclusion complexes, therefore lowering the dissolution temperature, as compared to palmitic acid due to higher steric interactions caused by the ascorbyl group.

Figure 4.6. DSC curves of inclusion complexes of amylose with a) ascorbyl palmitate, b) retinyl palmitate, or c) phytosterol ester, and d) amylopectin with retinyl palmitate.

In addition, Lebail and others (2000) proposed that in amylose-fatty acid complexes, the lipid could be in a \textit{trans-gauche} conformation resulting in part of the fatty acid outside the helix. These authors suggested that the presence of this conformation could depend on crystallization conditions and the necessity of locating the carboxylic head outside the helical structure. Similarly, it is possible that the ascorbyl head will be more favored to stay in the hydrophilic surrounding environment than the carboxylic group of palmitic acid, which may increase the necessity to locate this hydrophilic group outside the hydrophobic helical cavity, favoring a \textit{trans-gauche} conformation of the alkyl chain resulting in a shorter amylose helix. Figure 4.7a(i) shows an schematic representation of the proposed model of amylose-ascorbyl palmitate inclusion complexes.
Figure 4.7. Schematic representation of amylose inclusion complexes with a) ascorbyl palmitate, b) retinyl palmitate, and c) phytosterol ester in (i) the absence of native lipids or (ii) presence of native lipids. Figure shows a longer helix in the presence of native lipids due to the formation of a ternary complex. Palmitic acid was used as a representation of the native lipid.
Although retinyl palmitate also has a C16 hydrocarbon chain to form the inclusion complex with amylose, amylose-retinyl palmitate complexes showed a much lower dissociation temperature (68.9 ± 2.8 °C) as compared to amylose-ascorbyl palmitate complexes (Figure 4.6). The low dissociation temperature may be the result of shorter helices formed due to a much stronger steric effect caused by the large retinol molecule forming the ester with palmitic acid. The necessity of placing the large retinol molecule outside the amylose helix may also promote the trans-gauche conformation of the alkyl chain as described by Lebail and others (2000). In addition, the retinyl group may get entangled with the amylose chain, destabilizing the formation of a longer single helix. Figure 4.7b(i) shows an schematic representation of the proposed model of amylose-retinyl palmitate inclusion complexes.

Thermograms of amylose-phytosterol ester complexes showed a broad and flat endotherm from around 85 °C to 135 °C (Figure 4.6c). As described by the manufacturer, phytosterol esters were composed of approximately 60% plant sterols and 40% by weight canola oil fatty acids. Canola oil fatty acids are composed mainly of unsaturated fatty acids, approximately 62% of oleic (C18:1), 21% of linoleic (C18:2), and 8% of linolenic acid (C18:3) (Warner and others 1994). Therefore it would be expected that the variety of fatty acids used to form the phytosterol ester would result in different structures of inclusion complexes, such as helices with various lengths. The wide endotherm observed for amylose-phytosterol esters is in agreement with the dissociation temperature of amylose inclusion complexes with C18:1, C18:2, and C18:3 fatty acids, which are around 103–112 °C (Karkalas and others 1995; Tufvesson and others 2003b), 94–103 °C (Karkalas and others 1995; Lalush and others 2005; Tufvesson and others 2003b), and 87–95 °C (Karkalas and others 1995; Tufvesson and others 2003b), respectively. Karkalas and others (1995) also observed that the dissociation endotherm of mixed acid complexes was broad with a peak melting temperature between the melting temperatures of the monoacid complexes. The composition of phytosterol esters by mainly unsaturated fatty acids could have also contributed to the lower yield of the precipitate. Unsaturated fatty acids are less effective on forming inclusion complexes as
compared to their saturated counterparts (Hahn and Hood 1987; Karkalas and Raphaelides 1986; Zabar and others 2009).

DSC curves of amylose complexes with retinyl palmitate and phytosterol ester also showed an endotherm with peak temperature above 140 °C. In some replicates of ascorbyl palmitate samples, a small endotherm above 140 °C was also observed (data not shown). An endotherm around 150 °C has been previously reported when heating amylose-lipid inclusion complex samples. This high temperature endotherm has been attributed to the dissociation of retrograded amylose (Raphaelides and Karkalas 1988) or a structure formed by uncomplexed amylose chains (Biliaderis and Galloway 1989; Biliaderis and others 1985). Biliaderis and others (1985) showed that the temperature of this transition is affected by the sample’s water content. Thus, the observed high temperature endotherm may be attributed to a structure formed by uncomplexed amylose.

The amount of entrapped fatty acid ester was evaluated using FTIR. This technique has been previously used to quantify the amount of complexed compounds that contain a carbonyl group (Biais and others 2006; Fanta and others 1999a; Uchino and others 2002). Figure 4.8 shows the FTIR spectra of pure Hylon VII and fatty acid esters, their physical mixture, and their inclusion complexes. FTIR spectrum of amylose was similar to that of Hylon VII. As describe in section 4.2.5, the physical mixture was used to generate the standard curve for the estimation of the amount of entrapped ligand. For retinyl palmitate and phytosterol ester samples, the carbonyl peak in the complexes and physical mixture resembled the one of the pure compound. However, as shown in Figure 4.8a, the peaks corresponding to the carbonyl groups in ascorbyl palmitate complexes slightly shifted to a higher wave number. Thus, direct use of the values obtained using FTIR may not provide an accurate determination of the amount of entrapped ascorbyl palmitate.
Figure 4.8. FTIR spectra of (i) Hylon VII, (ii) Hylon II-fatty acid ester complex, (iii) Hylon VII-fatty acid ester physical mixture, and (iv) pure fatty acid esters in a) ascorbyl palmitate, b) retinyl palmitate, and c) phytosterol ester samples.
In order to evaluate the accuracy of the estimated entrapped values obtained with the FTIR, $^1$H NMR was also used to estimate the proportion of ascorbyl palmitate in the inclusion complexes. Based on the $^1$H NMR results, the FTIR method underestimated the amount of entrapped ascorbyl palmitate giving values that were an average of 35% of the values estimated with the NMR. Hence, the percentage of ascorbyl palmitate entrapped in the complexes estimated by the FTIR were corrected based on the NMR results ($\%$ ascorbyl palmitate in complex = FTIR value/0.35).

A shift of the carbonyl peak of the FTIR spectra has been previously reported for amylose complexes with salicylic acid analogues (Uchino and others 2002) and $p$-aminobenzoic acid (Tozuka and others 2006). Uchino and others (2002) attributed this shift to the breakage of hydrogen bonds between carboxyl groups of molecules of salicylic acid analogues in the crystalline state, so the guest molecules can be included inside the amylose helix. It is possible that a similar phenomenon occurs when ascorbyl palmitate crystals are dissolved and the individual molecules are included in the amylose helix. On the other hand, Tozuka and others (2006) attributed the shift of the carbonyl band to the formation of hydrogen bonds between the carbonyl group of the ligand and hydroxyl groups of amylose. It is possible that a similar interaction occurs between the carbonyl groups of ascorbyl palmitate and hydroxyl groups of amylose. However, steric constraints may prevent retinyl palmitate and phytosterol ester from forming these hydrogen bonds, which could also contribute to the lower complexation efficiency of these two molecules.

Ethanol washing (50% aqueous solution) of amylose-fatty acid (C8:0, C10:0, C12:0, C16:0) inclusion complexes has been shown to remove the free fatty acids located in amorphous areas without extracting the fatty acids included within the crystal (Biais and others 2006; Godet and others 1995). However, ethanol washing could not remove uncomplexed phytosterol esters. FTIR results of complexes before hexane washing showed a high variability in the amount of entrapped phytosterol esters, in particular at high concentrations. After hexane washing, the amount of phytosterol esters in the
precipitates decreased, as well as the variability of the measurements. On the other hand, no significant differences (p>0.05) were observed in the amount of retinyl palmitate in the precipitates before or after hexane washing.

The amount of complexed fatty acid esters in the precipitates differed among ligands. The maximum % fatty acid ester in the precipitate (w fatty acid ester/ w dry starch) was observed for ascorbyl palmitate (6.3%) followed by retinyl palmitate (1.42%) and by phytosterol ester (0.73%) (Table 4.1). The proportion of fatty acid chains in the guest molecules could be responsible for some of the observed differences. As described previously, the weight fraction of fatty acid in ascorbyl palmitate, retinyl palmitate, and phytosterol ester is 0.62, 0.49 and 0.40, respectively. However, differences in weight fractions are not large enough to solely account for the observed differences in the % fatty acid esters in the precipitates.

The smaller size of the ascorbyl palmitate molecule may allow the formation of more inclusion complexes within the same amylose chain, resulting in higher amount of ascorbyl palmitate entrapment per weight of amylose. Based on DSC curves, in addition to inclusion complexes, precipitates of retinyl palmitate and phytosterol esters also contained certain amount of uncomplexed amylose (peak around 140 °C in Figure 4.6b and c). After the precipitates were washed, this uncomplexed amylose most likely did not contain fatty acid esters, resulting in a lower % retinyl palmitate and phytosterol ester in the precipitates (w/w amylose). It is possible that phytosterol ester precipitates had a larger ratio of uncomplexed:complexed amylose than that of retinyl palmitate samples, which resulted in a lower percentage of entrapment. This is in agreement with the larger ratio of uncomplexed:complexed amylose transition enthalpies (3.6 J/g:6.5 J/g) in phytosterol ester precipitates compared to that of retinyl palmitate precipitates (1.7 J/g : 14 J/g).

Although ascorbyl palmitate showed a better complexing ability with amylose, ascorbyl palmitate did not induced amylopectin precipitation. Similarly, amylopectin did
not precipitate when processed with phytosterol esters, but precipitation was induced with retinyl palmitate. This precipitate showed a very weak V-pattern (Figure 4.5d) indicating the formation of inclusion complexes between amylopectin and retinyl palmitate. Evidence that amylopectin may interact with lipids or surfactants has been previously reported (Eliasson and Ljunger 1988; Evans 1986; Gudmundsson and Eliasson 1990; Hahn and Hood 1987; Huang and White 1993; Kim and others 1992; Nakazawa and Wang 2004; Villwock and others 1999). It could be argued that these inclusion complexes were formed with a small fraction of amylose or intermediate material contaminating amylopectin samples. However, if this was the case, inclusion complexes with ascorbyl palmitate would have also been observed. The precipitate of retinyl palmitate with amylopectin had a lower % crystallinity and % retinyl palmitate (w/w amylopectin) than its amylose counterpart. DSC curves showed a transition endotherm around 76.2 ± 1.9 (Figure 4.6d) which was not significantly different (α>0.05) than the peak temperature observed for the dissolution of amylose-retinyl palmitate complexes.

4.3.2. Inclusion complexes with Hylon VII starch and the effect of native lipids

Precipitates were obtained with native and defatted Hylon VII with and without addition of fatty acid esters (Table 4.2). As shown by the V-type X-ray diffraction pattern, all three fatty acid esters formed inclusion complexes with Hylon HVII starch regardless of the presence of native lipid (Figure 4.9).

Native high amylose maize starch contains approximately 0.964% monoacyl lipids (w/w dry starch) which are composed of free fatty acids and lysophospholipids. Palmitic acid, stearic acid, and linoleic acid are the main fatty acids present as free fatty acids and in phospholipids (Morrison 1988), which can form inclusion complexes with starch molecules. Without fatty acid ester addition, the yield of precipitates was around 10% and 20% for defatted and native Hylon VII starch, respectively. A 10% yield difference between precipitates of native and lipid-free high amylose maize starch has also been previously reported (Tapanapunnitikul and others 2008). Tapanapunnitikul and
others (2008) explained that starch can precipitate out of solution by three mechanisms: simple retrogradation, complex formation between starch and native lipids, and complex formation between starch and added guest molecules. In defatted Hylon VII samples without fatty acid ester addition, the obtained precipitates are likely retrograded starch. It is also possible that inclusion complexes were formed between starch and some native lipids that remained in the starch after defatting. However, thermal analysis of defatted Hylon VII precipitates without fatty acid ester addition did not show any transition that could be attributed to inclusion complexes. DSC curves only showed one endotherm above 150 °C (Figure B4 in Appendix B), which is associated with retrograded amylose (Raphaelides and Karkalas 1988).

![X-ray diffraction patterns](image)

**Figure 4.9.** X-ray diffraction patterns of precipitates obtained with defatted Hylon HVII (a, b, c, and d) and native Hylon VII (a’, b’, c’, and d’) with a) ascorbyl palmitate, b) retinyl palmitate, c) phytosterol esters, and d) no fatty acid ester.
Table 4.2. Yield, % crystallinity, % entrapped fatty acid ester, and dissociation temperature of native and defatted Hylon VII precipitates made with 10% retinyl palmitate, phytosterol ester, or ascorbyl palmitate (g/100 g starch)\(^1\).

<table>
<thead>
<tr>
<th>Fatty acid ester</th>
<th>Yield of precipitate (g precipitate/100 g starch+fatty acid ester)</th>
<th>% Crystallinity</th>
<th>% fatty acid ester in precipitate (w/w starch)(^2)</th>
<th>Dissociation peak temperature of inclusion complexes (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native Hylon VII</td>
<td>Defatted Hylon VII</td>
<td>Native Hylon VII</td>
<td>Defatted Hylon VII</td>
</tr>
<tr>
<td>Ascorbyl palmitate</td>
<td>57 ± 3 a</td>
<td>50 ± 3 b</td>
<td>30 ± 3 a</td>
<td>27 ± 2 a</td>
</tr>
<tr>
<td>Retinyl palmitate</td>
<td>24 ± 6 a</td>
<td>20 ± 6 a</td>
<td>26 ± 3 a</td>
<td>21 ± 2 a</td>
</tr>
<tr>
<td>Phytosterol ester</td>
<td>17 ± 3 a</td>
<td>7 ± 2 b</td>
<td>23 ± 7 a</td>
<td>21 ± 1 a</td>
</tr>
<tr>
<td>None (control)</td>
<td>20 ± 5 a</td>
<td>10 ± 1 b</td>
<td>10 ± 4</td>
<td>NA</td>
</tr>
</tbody>
</table>

\(^1\) For the same analysis (yield, % crystallinity, or % fatty acid in precipitate), mean ± standard deviation (n ≥ 2) followed by the same letter show no significant differences between native and defatted Hylon VII at \(\alpha=0.05\) as determined by analysis of variance.

\(^2\) Reported values of % ascorbyl palmitate in precipitate are the adjusted FTIR values.

NA=Data not available
In the case of native Hylon VII precipitates, in addition to the endotherm associated with retrograded amylose, thermograms showed an endotherm around 103 °C, which can be associated with type I amylose-lipid inclusion complexes (Karkalas and others 1995; Tufvesson and others 2003b). In a few of the measurements of native Hylon VII precipitates, endotherms around 70 °C and 125 °C could also be observed. The transition around 70 °C could be associated with amylopectin retrogradation. However, inclusion complexes between native lipids and amylopectin cannot be ruled out. Transition temperatures between 118–125 °C have been associated with type II amylose-lipid inclusion complexes (Tufvesson and others 2003b), so the endotherm around 125°C could be associated with these structures.

Even though DSC curves suggested the presence of retrograded amylose in the precipitates of both native and defatted Hylon VII, X-ray diffractograms showed an amorphous pattern (Figure 4.9d and d’). It is possible that packing of retrograded amylose double helices was not sufficient to form large enough crystals to be detected by the X-ray diffractometer.

Yield, % crystallinity, and % fatty acid ester in retinyl palmitate precipitates were not significantly affected by the presence of native lipids (Table 4.2). However, it appears that complexes formed in the presence of native lipids had a higher thermal stability. The dissolution temperature of defatted Hylon VII complexes was 80.2 ± 12.9 °C and was significantly different from the 102 ± 13.8 °C dissolution temperature of native Hylon VII-retinyl palmitate complexes. Tapanapunnitikul and others (2008) suggested that native lipids may form ternary complexes between starch, guest molecule, and native lipids. These authors explained that native lipids may induce complex formation with the starch more avidly than low solubility flavor compounds. They proposed that a stable amylose-lipid complex could induce the formation of a more extended complex with the flavor molecules (Tapanapunnitikul and others 2008). Similarly, it is possible that native lipids formed a ternary complex with Hylon VII and
retinyl palmitate, inducing the formation of a longer helix with higher thermal stability (Figure 4.7b).

Complexes formed with ascorbyl palmitate or phytosterol ester also had significantly higher dissolution temperature when native lipids were present (Table 4.2). Similar to retinyl palmitate samples, it is possible that the formation of co-inclusion complexes of starch-fatty acid ester-native lipid resulted in longer helices with higher thermal stability (Figure 4.7a and c).

The yield of ascorbyl palmitate and phytosterol ester precipitates was significantly higher in the presence of native lipids (Table 4.2). The differences in yield were 7.6% and 10.3% for ascorbyl palmitate and phytosterol ester samples, respectively. As reported above, the yield difference between native and defatted Hylon VII was 10% due to the formation of starch-native lipid complexes. Hence, the formation of isolated starch-native lipid inclusion complexes could be the reason of the higher yield observed in ascorbyl palmitate- and phytosterol ester-native Hylon HVII precipitates. However, if that was the case, the % fatty acid ester in the precipitate would be lower due to a dilution effect. As suggested by Tapanapunnitikul and others (2008) complex formation with certain compounds may be enhanced by the formation of ternary complexes with high amylose maize starch native lipids. Thus, native lipids may not only increase the thermal stability of the complexes, but also, the lipids may increase the partition of these fatty acid esters into the starch helix, resulting in higher amount of inclusion complexes.

In general, the % fatty acid ester in native and defatted Hylon VII inclusion complexes was lower as compared to their amylose counterparts. It is possible that fatty acid ester molecules in the reaction medium get entangled with branched amyllopectin molecules, reducing the amount of ligand available to form the complexes with amylose, decreasing the encapsulation efficiency.
4.3.3. Effect of amount of added fatty acid ester

Precipitates were obtained in all samples and their yield (g precipitate/100 g Hylon VII+fatty acid ester) varied depending on the type of fatty acid ester and concentration used (Figure 4.10).

As shown in Figure 4.10, the yield of precipitates made with ascorbyl palmitate or retinyl palmitate initially increased as higher amount of guest molecules were added. The maximum yield of ascorbyl palmitate and retinyl palmitate precipitates was reached when 15% and 10% fatty acid esters were added, respectively. Above this concentration, higher amounts of added fatty acid esters resulted in a lower yield. For phytosterol ester precipitates, the maximum yield was observed at the lowest concentration used (2.5%) and appears to decrease above 10% added phytosterol ester (Figure 4.10).

![Figure 4.10. Yield of native Hylon VII precipitates with various concentrations of ascorbyl palmitate, retinyl palmitate, or phytosterol esters. Error bars represent the standard deviation.](image-url)
The decrease in yield observed for all types of fatty acid esters above a given amount of added compound could be due to a disruptive effect of high concentration of free fatty acid esters towards helical association. Lebail and others (2000) proposed that at high free fatty acid content, lipids can get trapped in the long polymer amylose chains without forming a complex. Similarly, the excess fatty acid esters can interact with polymer chains without forming a complex, possibly by physical sorption or association, which may reduce the availability of amylose segments and guest molecules to form inclusion complexes. Based on the size of these molecules, retinyl palmitate and phytosterol ester are more likely to get trapped in polymer chains, and therefore, the disruptive effect toward complex formation possibly occurs at a lower concentration.

Similar to amylose-fatty acid ester complexes, the highest yield was always observed for ascorbyl palmitate samples, followed by retinyl palmitate, and then by phytosterol ester samples. This trend was observed at all studied concentrations. As discussed above, the differences in yield obtained among guest molecules can be explained by the difference in the ability of the ligand to form inclusion complexes with starch and its solubility.

As described previously, Godet and others (1995b) reported a yield of 96.6% complexed amylose (DP 900) when 10% palmitic acid (w/w amylose) was used for complexation. The maximum yield for native Hylon VII-ascorbyl palmitate samples was only 65.6% (g precipitate/ 100 g Hylon VII + fatty acid ester) when 15% ascorbyl palmitate was added. Taking into account that the weight fraction of palmitic acid in ascorbyl palmitate is 0.62, then the proportion of added palmitic acid was around 9%. Thus, the lower yield of Hylon VII-ascorbyl palmitate complexes was not only caused by the lower amount of added fatty acid. Based on the manufacturer, Hylon VII starch contains 70% amylose. As shown in Table 4.1, precipitates of Hylon VII amylopectin-ascorbyl palmitate were not obtained indicating that Hylon VII amylopectin could not form complexes with ascorbyl palmitate. Therefore, the lower yield of Hylon VII-
ascorbyl palmitate complexes was caused by the lower proportion of amylose used during processing.

**X-ray diffraction patterns, percentage of crystallinity, and relative crystal size**

Precipitates from all three fatty acid esters at all studied concentrations had a V-type diffraction pattern (Figure 4.11) indicating the formation of inclusion complexes. The percentage of crystallinity of the precipitates varied depending on the guest molecule and concentration used (Figure 4.12). The % crystallinity of ascorbyl palmitate samples increased as more ascorbyl palmitate was added in the reaction medium. The % crystallinity of retinyl palmitate precipitates appeared to reach a maximum at around 10–15% added retinyl palmitate. At 20% added retinyl palmitate, the % crystallinity decreased to about 20%, and higher addition of retinyl palmitate did not seem to significantly affect the % crystallinity. This trend of % crystallinity and the trend of yield (Figure 4.10) of the obtained precipitates as a function of added retinyl palmitate was similar. These results suggest that in retinyl palmitate samples, a higher % crystallinity resulted in precipitation of higher amounts of inclusion complexes. On the other hand, it appears that the % crystallinity of phytosterol ester precipitates was not significantly affected by the amount of phytosterol ester added during processing.

The relative crystal size estimated from the broadening of the peak at around 13° appears to be larger when complexes were produced with lower proportion of fatty acid esters (Figure 4.13a). The estimated size from the 2θ ≈13° peak was generally larger than that from the 2θ ≈20° peak (Figure 4.13). Previous studies (Le Bail and others 2005; Rondeau-Mouro and others 2004; Yamashita 1965) have reported that the peak at 2θ ≈13° corresponds to the (130) plane of the orthorhombic unit cell of V_{6} crystals. For anhydrous n-butanol complexes, Yamashita (1965) observed no difference in the d-spacings of the (130) and (200) planes because of the pseudo-hexagonal nature of the unit cell of this complex, and therefore the reflection peak at 2θ ≈13° corresponded to these two planes. The same author also suggested that the growth rate in the (100) plane is
Figure 4.11. X-ray diffraction patterns of precipitates made from native Hylon VII and various concentrations of (a) ascorbyl palmitate, (b) retinyl palmitate, and (c) phytosterol esters. Percentages shown on graphs represent the % added fatty acid ester (w/w Hylon VII).
Figure 4.12. Percentage of crystallinity of precipitates made from native Hylon VII and various concentrations of (a) ascorbyl palmitate, (b) retinyl palmitate, and (c) phytosterol esters. Error bars represent the standard deviation.
Figure 4.13. Relative crystal size estimated from the broadening of the main peak reflection at a) $2\theta \approx 13^\circ$ and b) $2\theta \approx 20^\circ$ of the X-ray diffraction pattern. Error bars represent the standard deviation.
faster than that in the (010) plane, and therefore, the longer side of the rectangular crystalline lamellae corresponds to the $a$ axis of the unit cell. Similarly, it is possible that in the present study, the longer side of the crystals corresponded to the $a$ axis of the unit cell, and the reflection peak at $2\theta \approx 13^\circ$ corresponded to both the (130) and (200) planes, resulting in a larger estimated relative crystal size as compared to that from the $2\theta \approx 20^\circ$ peak.

The relative crystal size estimated from the $2\theta \approx 20^\circ$ reflection peak of retinyl palmitate and phytosterol ester precipitates was not significantly affected ($p>0.05$) by the % added fatty acid ester during processing. On the other hand, the relative crystal size of ascorbyl palmitate precipitates linearly increased ($R^2=0.74$) as the % added ascorbyl palmitate increased to 50% (Figure 4.13). At the highest ascorbyl palmitate concentration, the relative crystal size was roughly double the size of the crystals at low ascorbyl palmitate concentrations.

**Amount of complexed fatty acid esters**

The amount of entrapped fatty acid ester varied depending on the type of fatty acid ester. The general trend for the three evaluated compounds was an increase in the entrapment of fatty acid esters in the precipitate as the amount of added guest molecules increased. At high concentrations of fatty acid esters, the % entrapped guest molecules seems to reach a plateau (Figure 4.14). The increase in the amount of entrapped compounds as the amount of added guest molecules increased suggests that saturation of amylose chains were not a requisite for precipitation. This is contrary to the observations by Raphaelides and Karkalas (1988) where saturated amylose chains were removed preferentially from solution as compared to amylose chains partially forming inclusion complexes with fatty acids.

The amount of ascorbyl palmitate included in the complexes increased in a non-linear manner with higher proportions of added fatty acid ester, following a second order
Figure 4.14. Amount of fatty acid ester entrapped in precipitates of native Hylon VII with a) ascorbyl palmitate, b) retinyl palmitate, or c) phytosterol ester. Error bars represent the standard deviation.
polynomial equation ($R^2=0.91$) within the range of studied concentrations. Not all of the added ascorbyl palmitate was included in the complexes, not even at the lowest concentration used. As shown in Figure 4.14a, in ascorbyl palmitate precipitates, it appears that the saturation of complexes was almost reached at 50% added ascorbyl palmitate, when precipitates contained around 10% ascorbyl palmitate (w/w Hylon VII). This corresponds to approximately 9 g palmitic acid per 100 g amylose considering 0.62 the weight fraction of palmitic acid in ascorbyl palmitate, and 70% amylose content in Hylon VII starch.

For amylose-palmitic acid complexes, the saturation molar ratio has been calculated to be 16.7 AGU per fatty acid molecule based on the total length of the fatty acid and assuming 6 AGU per helical turn with a pitch of 0.8 nm (Karkalas and Raphaelides 1986). However, only the alkyl chain is located inside the helix (Godet and others 1995b). Thus, based on the 2.014 nm length of the fatty acid alkyl chain (Karkalas and Raphaelides 1986), the saturation molar ratio would be 15.1 AGU per fatty acid molecule. Because ascorbyl palmitate is a monoester of palmitic acid with ascorbic acid, then the saturation molar ratio for ascorbyl palmitate is also 15.1 AGU per ascorbyl palmitate molecule. This molar ratio corresponds to 17 % ascorbyl palmitate (w/w AGU). Therefore, even at 50% added ascorbyl palmitate, the % ascorbyl palmitate in the precipitate did not reach the calculated saturation concentration.

When 50% ascorbyl palmitate were added in the reaction medium, and the precipitates contained approximately 10% ascorbyl palmitate, 24 glucose residues were needed to form an inclusion complex with one molecule of ascorbyl palmitate (Table 4.3). As proposed in section 4.3.1, amylose-ascorbyl palmitate complexes formed helices shorter than those of amylose-palmitic acid complexes. Thus, less than 15.1 AGU are actually forming the helix, and the remaining glucose residues are forming the amorphous region.
Table 4.3. Stoichiometry (mols AGU/mol fatty acid ester) of native Hylon VII-fatty acid ester inclusion complexes at various concentrations of added fatty acid ester.

<table>
<thead>
<tr>
<th>% added fatty acid ester</th>
<th>Ascorbyl palmitate</th>
<th>Retinyl palmitate</th>
<th>Phytosterol ester¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>536</td>
<td>1600</td>
<td>1505</td>
</tr>
<tr>
<td>5.0</td>
<td>92</td>
<td>1971</td>
<td>1430</td>
</tr>
<tr>
<td>10</td>
<td>64</td>
<td>498</td>
<td>2396</td>
</tr>
<tr>
<td>15</td>
<td>55</td>
<td>295</td>
<td>478</td>
</tr>
<tr>
<td>20</td>
<td>34</td>
<td>348</td>
<td>698</td>
</tr>
<tr>
<td>50</td>
<td>24</td>
<td>65</td>
<td>541</td>
</tr>
</tbody>
</table>

¹ Calculated based on the molecular weight of sitosterol oleate

High variability of the % entrapped retinyl palmitate and phytosterol ester was observed. Statistical analysis showed that the replicate had a significant effect on the % entrapped compound (p<0.05). Therefore, replicate was also included as a source of variation during the analysis. Based on regression analysis, a logarithmic transformation of the % of entrapped fatty acid ester was necessary to obtain a valid quadratic model for both retinyl palmitate (R²=0.81) and phytosterol ester (R²=0.85). Figure 4.14b and c show the % retinyl palmitate and % phytosterol in precipitates after logarithmic transformation (untransformed data is shown in Appendix B). In precipitates made with retinyl palmitate and phytosterol ester, the amount of guest molecules seems to reach a plateau around 15–20% added fatty acid ester (Figure 4.14b and c), likely due to the solubility limit of these molecules in the reaction medium.

A large proportion of the added fatty acid esters did not form complexes with the starch, even at low fatty acid ester:starch ratios. This is in agreement with previous studies in which free lipids were detected after palmitic acid (Raphaelides and Karkalas 1988) and monoglyceride (Eliasson and Krog 1985) were complexed with amylose in a DMSO solution, even when the ratio amylose:lipid was increased. Similarly, Lebail and others (2000) observed that not all the 10% added palmitic acid (w/w amylose) formed inclusion complexes with amylose. Competition between DMSO and lipid molecules to occupy the helical cavity, (Raphaelides and Karkalas 1988), and kinetic and steric factors...
(Le Bail and others 2000) have been suggested as possible reasons why 100% complexation is not achieved. Le Bail and others (2000) further explained that amylose chains can trap the excess lipids without forming a complex, which is less probable to happen at lower fatty acid concentration when there is no excess fatty acid. Based on the water solubility and molecular structure of the ligands, the large proportion of uncomplexed retinyl palmitate and phytosterol ester is not surprising. The larger molecules linked to the lipid alkyl chain in retinyl palmitate and phytosterol esters are more likely to get trapped in amylose chains, resulting in less amylose chains available to form helical structures, which may explain the larger proportion of these fatty acid esters that remained uncomplexed. In the case of ascorbyl palmitate, in addition to the interaction of molecules with amylose chains at high ligand concentration, ascorbyl palmitate most likely partitioned between the DMSO solution and amylose helices.

**Thermal analysis**

Native Hylon VII-ascorbyl palmitate complexes disassociated in a temperature range between 85 °C to 130 °C, with a peak temperature around 99–103 °C (Figure 4.15a). At lower ascorbyl palmitate concentrations, the peak disassociation temperature was around 100–101 °C. As the concentration of ascorbyl palmitate increased, the shape of the endotherm changed, and the appearance of a second peak around 102–103 °C suggested the presence of a second population of structures with slightly higher thermal stability. The higher dissociation temperature suggests the formation of a longer helical structure. In samples made with 50% ascorbyl palmitate, only a peak at around 102–103 °C could be observed. Multiple endotherms of type II complexes have also been previously reported (Biliaderis and Galloway 1989; Biliaderis and others 1985; Karkalas and others 1995).

Godet and others (1996) proposed that in amylose palmitic acid complexes, two palmitic acid molecules arranged tail to tail are included in the helix. It is possible that at
Figure 4.15. DSC thermograms of precipitates made from native Hylon VII and various concentrations of (a) ascorbyl palmitate, (b) retinyl palmitate, and (c) phytosterol esters.
low concentration of ascorbyl palmitate, shorter helices with a lower dissociation
temperature containing only one ascorbyl palmitate molecule were forming the crystals.
As the concentration of ascorbyl palmitate increased, longer helices with higher thermal
stability were formed possibly due to the inclusion of two ascorbyl palmitate molecules
arranged tail to tail within the same helix, similar to the model proposed by Godet and
others (1996) for amylose-palmitic acid complexes.

Higher dissociation enthalpies were observed in samples made with higher %
ascorbyl palmitate. The increase in dissociation enthalpy with ascorbyl palmitate
concentration is in agreement with previous studies of amylose-palmitic acid complexes
prepared using the DMSO method, however, in that investigation, the dissolution
temperature was not affected by palmitic acid concentration (Raphaelides and Karkalas
1988). Raphaelides and Karkalas (1988) explained that lower dissociation enthalpies are
caused if part of the amylose remains uncomplexed. The lower dissociation enthalpy at
lower ascorbyl palmitate concentration suggests that at these low concentrations partially
complexed amylose chains precipitated. These results are in agreement with the lower %
crystallinity (Figure 4.12) and higher AGU/mol compound (Table 4.3) of samples made
with lower % ascorbyl palmitate.

Thermal analysis of retinyl palmitate inclusion complexes showed high variability
in their thermal behavior between replicates and within samples. Individual endotherms
at various temperatures could be differentiated. In some measurements, these
endotherms seemed to overlap forming a broad endotherm (Figure 4.15b). These results
suggest the formation of helical structures with various thermal stabilities. In general
three endotherms could be observed: a low temperature endotherm around 60–80 °C,
medium temperature endotherm around 90–102 °C, and high temperature endotherm
around 140–150 °C. The high temperature endotherm 140–150 °C can be attributed to
the dissociation of a structure formed by uncomplexed amylose (e.g. retrograded
amylose) (Biliaderis and others 1985). As reported in section 4.3.1 and 4.3.2, the
endotherm around 60–80 °C is due to the dissolution of starch-retinyl palmitate
complexes, while the endotherm around 90–102 °C may be associated with the
dissolution of co-inclusion complexes of starch with retinyl palmitate and native lipids.
In general, at low concentrations, only the endotherm around 90–102 °C could be
observed (Figure 4.15b). It is possible that at low added retinyl palmitate, all the
complexes formed are in the form of co-inclusion complexes with native lipids.
However, as more retinyl palmitate is added, shorter starch-retinyl palmitate complexes
are formed in addition to the co-inclusion complexes.

Similarly, thermograms of phytosterol ester complexes showed high variability in
the thermal behavior of the samples. This variability was observed even within the same
sample. Multiple endothermic peaks could be identified, which sometimes overlapped
(Figure 4.15c). Low temperature endotherms showed a peak temperature around 60–70
°C, medium temperature endotherms varied between 94–126 °C, and the high
temperature endotherm had a peak temperature above 138 °C. Medium temperature
endotherms (peak temperature between 104–126 °C) are in agreement with the melting
transition observed for amylose-phytosterol complexes (Figure 4.6), while the endotherm
above 138 °C is likely associated with structures formed by uncomplexed amylose. A
thermal transition around 60 °C observed in amylose-palmitic acid complexes has been
attributed to melting of excess palmitic acid outside the helix (melting point of palmitic
acid = 62.7 °C) (Raphaelides and Karkalas 1988). However, in the present study, the low
temperature transition did not represent the melting of uncomplexed phytosterol esters
because DSC analysis of the pure compound showed that melting occurred below 60 °C.
Thus, given the variety of fatty acids and sterols that forms phytosterol esters, it could be
possible that some of the inclusion complexes are formed as shorter helices. It is also
possible, that this low temperature endothermic transition represents the dissociation of
type I inclusion complexes.
4.3.4. Summary

Ascorbyl palmitate

When processing Hylon VII starch and ascorbyl palmitate to produce inclusion complexes, the linear fraction (i.e. amylose) forms the helical inclusion complexes. At low concentrations of added ascorbyl palmitate, crystalline inclusion complexes are formed, but only part of the polymer chain forms the complexes. As more ascorbyl palmitate is added, more guest molecules are available to form the complexes, resulting in higher complexation and yield of precipitates. The maximum yield is obtained when 15% ascorbyl palmitate (w/w starch) is added to the reaction medium. However, at this maximum yield, saturation of the complexes is not reached. Above this concentration, the excess ascorbyl palmitate molecules in the reaction medium possibly interact with starch molecules without forming inclusion complexes or disrupting the formation of helices, which results in a decrease in the amount of starch that precipitates, and thus, a lower yield. As more ascorbyl palmitate is added, the relative crystal size, % crystallinity, thermal stability, and the % entrapment increase. It is possible that at higher concentrations of ascorbyl palmitate in the reaction medium, two ascorbyl palmitate molecules arranged tail to tail form a complex with the amylose helix, forming a longer helix with higher thermal stability (Figure 4.16). Inclusion complexes with ascorbyl palmitate appeared to approach saturation when 50% ascorbyl palmitate was added. However, at this % added ascorbyl palmitate, the % of entrapment (10.5%) was much lower than the calculated saturation limit (17%).

Retinyl palmitate

Hylon VII starch forms inclusion complexes with retinyl palmitate. Both amylose and amylopectin can form these inclusion complexes, although the ability of amylopectin to form complexes is low. Maximum yield of retinyl palmitate precipitates was observed at 10% added compound (w/w starch). Yield appears to slightly decrease above this
Figure 4.16. Schematic representation of the proposed formation of Hylon VII-ascorbyl palmitate inclusion complexes at (a) low % added ascorbyl palmitate, (b) 15% added ascorbyl palmitate when maximum yield is observed, and (c) 50% added ascorbyl palmitate.
concentration (10% added compound) possibly caused by a disruption effect towards helical formation of high amounts of retinyl palmitate molecules in the solution. At low retinyl palmitate concentration, only co-inclusion complexes with native lipids may be formed. At higher concentrations, in addition to the co-inclusion complexes, shorter starch-retinyl palmitate complexes may be produced, which have lower thermal stability and lower packing ability resulting in lower % crystallinity. The higher amount of entrapped retinyl palmitate at higher concentrations of added compound suggests that the lower crystallinity is not due to a larger proportion of uncomplexed amylose in the precipitate, but it is possibly caused by the presence of type I inclusion complexes.

Phytosterol ester

Hylon VII starch forms inclusion complexes with phytosterol esters. These inclusion complexes are formed by amylose and not by amylopectin. As more phytosterol ester was added in the reaction medium, less precipitate (lower yield) containing more phytosterol ester (higher % of entrapped phytosterol ester) was produced. Both yield and % entrapped compound seem to reach a plateau at 30% added compound. The decrease in yield at higher phytosterol ester concentrations could be caused by a possible association of guest molecules with starch without forming a complex, resulting in less available amylose for complexation or retrogradation. The higher % entrapment at high phytosterol ester addition may be the result of a lower proportion of retrograded amylose in the precipitate. The % crystallinity did not seem to be significantly affected by the amount of added ligand.

4.4. Conclusions

Selected fatty acid esters of bioactive compounds (ascorbic acid, retinol, and phytosterols) formed inclusion complexes with amylose. However, only retinyl palmitate formed complexes with amylopectin. Hylon VII starch could be used to form these inclusion complexes. In general, native lipids increased the yield of the precipitates and
the thermal stability of the complexes, possibly caused by the formation of co-inclusion complexes of starch-fatty acid ester-native lipid. Formation of starch inclusion complexes with fatty acid esters seem to be limited to the solubility of the compound in the reaction medium and the structure of the molecule forming the ester with the fatty acid.

Thus, Hylon VII-fatty acid ester inclusion complexes may have potential for the delivery of bioactive compounds. However, the formation of these complexes may be limited by the solubility and molecular structure of the specific compound forming the ester with the fatty acid.

4.5. References


Chapter 5
FORMATION OF STARCH SPHERULITES IN THE PRESENCE OF FATTY ACID ESTERS OF BIOACTIVE COMPOUNDS

5.1. Introduction

Interest in many bioactive compounds and their use in foods is growing in large part because of the apparent health benefits of these molecules. For example, phytosterols, also called plant sterols, are well known to reduce low density lipoprotein-cholesterol levels, lowering the risk of cardiovascular diseases (Moreau and Hicks 2004). However, addition of some of these compounds into food products is a technological challenge, because they are easily degraded during processing, storage, or their passage through the gastrointestinal tract after consumption (Lesmes and others 2008; Shimoni 2009). Many bioactive compounds, such as some phytonutrients, are bitter, acrid or astringent (Drewnowski and Gomez-Carneros 2000), which may negatively affect the sensory properties of the food products. Moreover, their solubility in water is often limited and, therefore, a carrier to increase their dispersability in a hydrophilic food environment may be necessary. Encapsulation of bioactive compounds may increase their stability during processing, storage and in the gastrointestinal tract after consumption, releasing these molecules in the small intestine where they are absorbed into the blood stream (Shimoni 2009). Addition of vitamins in food products is a common practice in the food industry. Thus, methods to improve their stability during processing, storage, and after consumption are of great interest.

Starch and starch based ingredients (e.g. modified starches, maltodextrins and cyclodextrins) are widely used for encapsulation purposes (Madene and others 2006). Their low cost, availability, biodegradability, food grade (Öngen and others 2002), and physicochemical properties, including retrogradation, film forming ability, complex
formation, and resistance to amylase hydrolysis at different degrees, make starch and starch based ingredients promising materials for delivery systems.

The ability of amylose to form inclusion complexes with some low molecular weight polar and non-polar substances, such as iodine, alcohols, fatty acids, and aromas is well known (Rondeau-Mouro and others 2004). Recently, this ability of amylose has been investigated as a method for molecular encapsulation of non-drug bioactive compounds (Cohen and others 2008; Lalush and others 2005; Lesmes and others 2008; Lesmes and others 2009; Zabar and others 2009), including conjugated linoleic acid (Lalush and others 2005; Yang and others 2009) and genistein (Cohen and others 2008). The results from these studies suggested that the encapsulated compounds can be protected from the acidic environment of the stomach and be released enzymatically in the small intestine (Lalush and others 2005; Yang and others 2009), where these compounds can be absorbed into the blood stream. Other bioactive compounds that can form inclusion complexes with amylose and high amylose maize starch are ascorbyl palmitate, retinyl palmitate, and phytosterol esters (See Chapter 4).

Previous studies have shown that starch inclusion complexes with fatty acids (Bhosale and Ziegler 2009; Byars and others 2003, 2006; Fanta and others 2002; Fanta and others 2005; Fanta and others 2006; Peterson and others 2005; Shogren and others 2006) or some flavors (Heinemann and others 2003; Heinemann and others 2005) can induce spherulitic crystallization of starch, forming small torus-shape and large spherical/lobed particles. Heinemann and others (2003) reported that these spherulites are slowly hydrolyzed at low concentrations of α-amylase, while breakdown of spherulites occurs at high α-amylase concentration, suggesting that they may be slowly degraded in the mouth and completely degraded in the gastrointestinal tract.

Another type of starch spherulite can be formed after heating a starch dispersion to temperatures above 170 °C and quickly cooling it to temperatures below 70 °C (Creek and others 2006). These spherulites have a round/spherical morphology and amylose
inclusion complexes are not required for their formation (Creek 2007; Creek and others 2006; Nordmark and Ziegler 2002a, 2002b; Suwanayuen 2009; Ziegler and others 2005; Ziegler and others 2003). Previous research showed via confocal scanning laser microscopy successful matrix encapsulation of fluorescein dye in this type of round spherulites (Ziegler, personal communication, May 14, 2008).

Bhosale and Ziegler (2009) showed that the round spherulites contained 28% resistant starch, while the torus-shape spherulites contained 39% resistant starch. After amylase hydrolysis, pores and an uneven surface were observed in round spherulites, while torus-shape spherulites were degraded with some evidence of recrystallization. These authors reported that morphology of the spherulites and entrapment of guest molecules in these particles depended on the processing conditions. Thus, they suggested that by choosing proper processing conditions, starch spherulites may be tailored for controlled- or targeted-release delivery systems.

Given the ability of starch spherulites to entrap certain compounds, and the effect of processing parameters on spherulite formation and guest molecule entrapment, the objectives of the present study were to a) evaluate the ability of spherulites to form in the presence of fatty acid esters and entrap ascorbyl palmitate, retinyl palmitate, and phytosterol esters, and b) evaluate the effect of processing conditions on spherulite formation and fatty acid ester entrapment.

5.2. Materials and Methods

5.2.1. Materials

High amylose maize starch (Hylon VII) was supplied by National Starch and Chemical Company (Bridgewater, NJ, USA). The fatty acid esters evaluated were ascorbyl palmitate, retinyl palmitate, and phytosterol esters. Ascorbyl palmitate and
retinyl palmitate were acquired from Sigma-Aldrich, Inc (St. Louis, MO, USA). Phytosterol esters were CoroWise™ plant sterol esters donated by Cargill Incorporated (Wayzata, MN, USA).

5.2.2. Sample preparation

In a 4.5 mL glass vial, 4.3 mL of a 2%, 5%, 8%, or 10% Hylon VII starch aqueous dispersion (w/w) was prepared (See section 5.2.3). The vial was covered with an aluminum foil disc (13 mm diameter and 0.11 mm thickness) and tightly sealed using a black phenolic screw cap with a PTFE/silicone/PTFE septum. The aluminum foil disc was necessary to prevent suction of the septum into the vial during processing. The glass vial was placed into a stainless steel pressure vessel equipped with a thermocouple (SICSS-062U-6-SHX, Omega Engineering, Inc., Stamford, CT, USA) and a cable coil heater (125CH24A1-X, Watlow, St. Louis, MO, USA) connected to a temperature controller (CNi32 temperature and process controller, Omega Engineering, Inc., Stamford, CT, USA) (Figure 5.1). The pressure vessel was built using a 9 cm 304 stainless steel tube (19.05 mm outside diameter and 1.651 mm wall thickness) and Swagelok® fittings (Swagelok Company, Solon, OH, USA). The thermocouple was located in the pressure vessel in such a way that when closing the vessel, the probe of the thermocouple went through the septum and the end of the probe was located at approximately the center of the glass vial (Figure 5.1). Before closing the pressure vessel, the space outside the glass vial was filled up with water to improve the heating process.

Samples were heated to the desired heating temperature ($T_{\text{heat}}$) using the coil heater. Immediately after heating, the pressure vessel was placed into a circulating oil bath with a programmable temperature controller (model 1147P, VWR International, USA) pre-heated to a given intermediate temperature ($T_{\text{in}}$). When the sample temperature reached 90 °C, the vessel was carefully opened without removing the thermocouple probe from the glass vial, and an ethanolic solution with the desired
amount of fatty acid ester was injected in the vial using a syringe (Hamilton® 750 RN syringe, Reno, NV, USA). To improve the dispersion of the fatty acid ester solution into the sample, injection started at the bottom of the vial and the syringe was pulled towards the top of the vial as the solution was injected. After injection, the pressure vessel was closed, shaken manually, and replaced inside the oil bath. Samples were cooled to the final temperature ($T_{cool}$) at a specific cooling rate using the programmable circulating oil bath. After cooling, the glass vial containing the sample was kept at room temperature overnight. Samples were collected by centrifugation, washed three times with 15 mL ethanol, and dried in a desiccator until used.

Figure 5.1. Schematic representation of the pressure vessel equipped with a thermocouple and a coil heater connected to a temperature controller used to prepare the samples

Fractionation of samples was based on particle size before ethanol washing. Samples were dispersed in excess water and separated by sedimentation of the larger particles, leaving the small particles suspended in the dispersion (Fanta and others 2006, 2008; Peterson and others 2005).
5.2.3. Experimental design

*Formation of Hylon VII spherulites in the presence of fatty acid esters*

In order to evaluate the ability of Hylon VII starch to form spherulites and entrap ascorbyl palmitate, retinyl palmitate, and phytosterol esters, a 10% Hylon VII starch dispersion (w/w dispersion) was processed as described in section 5.2.2 using the processing conditions showed in Table 5.1. For this part of the study, the amount of added fatty acid ester was 10% (w/w Hylon HVII).

Table 5.1. Processing conditions to evaluate the ability of Hylon VII starch to form spherulites and entrap ascorbyl palmitate, retinyl palmitate, and phytosterol esters.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$T_{\text{heat}}$ (°C)</th>
<th>$T_{\text{int}}$ (°C)</th>
<th>Cooling rate (°C/min)</th>
<th>$T_{\text{cool}}$ (°C)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>180</td>
<td>120</td>
<td>1</td>
<td>40</td>
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<tr>
<td>3</td>
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</table>

*Effect of processing parameters on spherulite formation and entrapment of ascorbyl palmitate*

The effect of Hylon VII concentration (% Hylon VII as g dry Hylon VII/100 g dispersion), added ascorbyl palmitate concentration (% added ascorbyl palmitate as g ascorbyl palmitate/100 g amylose), $T_{\text{heat}}$, holding time at the $T_{\text{int}}$ of 90 °C after addition of fatty acid esters, cooling rate, and $T_{\text{cool}}$ on spherulite formation and entrapment of ascorbyl palmitate were studied following a quarter fractional factorial design ($2^{n-2}$) with three center points (Table 5.2). Experiments were completely randomized. Data was analyzed using MODDE statistical software (version 7.0.0.1, Umetrics, Umeå, Sweden).
Table 5.2. Fractional factorial design and observed values for yield of the precipitates (% yield), % entrapped ascorbyl palmitate in the precipitate, and dissolution enthalpy of Hylon VII-ascorbil palmitate samples ($\Delta H_1$)

<table>
<thead>
<tr>
<th>Exp No</th>
<th>Run No</th>
<th>% Hylon VII (w/w dispersion)</th>
<th>% added ascorbyl palmitate, (w/w amylose)</th>
<th>$T_{\text{heat}}$ (°C)</th>
<th>Holding time at $T_{\text{int}} = 90$ °C (min)</th>
<th>Cooling rate (°C/min)$^2$</th>
<th>$T_{\text{cool}}$ (°C)</th>
<th>% Yield (w/w added Hylon VII + ascorbyl palmitate)</th>
<th>% entrapped ascorbyl palmitate (w/w Hylon VII)</th>
<th>$\Delta H_1$ (J/g)</th>
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<tr>
<td>1</td>
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<td>2</td>
<td>140</td>
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<tr>
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<td>25</td>
<td>50.4</td>
<td>1.38</td>
<td>3.9</td>
</tr>
</tbody>
</table>

$^1$ % added ascorbyl palmitate (g ascorbyl palmitate/100 g amylose) considering 70% amylose in Hylon VII

$^2$ A logarithmic transformation of cooling rate was used in the analysis: Log (0.04) = -1.4, Log(0.2) = -0.7, and Log(1) = 0

nd = not determined due to insufficient amount of sample
5.2.4. Light microscopy

Immediately after opening the glass vial or after fractionation, samples were observed using a BX41 light microscope equipped with polarizing filters (Olympus, Edgemont, PA, USA) connected to a digital camera (Spot Insight QE camera, Diagnostic instrument, USA).

5.2.5. Wide angle X-ray diffraction

Wide angle X-ray diffraction patterns were obtained with a Rigaku MiniFlex II desktop X-Ray diffractometer (Rigaku Americas Corporation, TX, USA) as described in Section 4.2.3.

5.2.6. Thermal analysis

Approximately 5 mg of dry sample were weighed into a 60 µL stainless steel DSC pan (Perkin-Elmer Instruments, Norwalk, CT, USA) and water was added to obtain a 10% dispersion (w/w). Samples were analyzed as described in Section 4.2.4.

5.2.7. Determination of presence of fatty acid esters by Fourier transformed infrared spectroscopy

The presence of fatty acid esters in the samples was evaluated using diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) with a Bruker IFS 66/S FTIR Spectrometer (Bruker Optics, Billerica, MA) from 500 cm\(^{-1}\) to 4000 cm\(^{-1}\) at a resolution of 6 cm\(^{-1}\). The carbonyl peak was used as an indication of the presence of the fatty acid esters in the sample. For retinyl palmitate and phytosterol ester, the carbonyl
peak was around 1738 cm$^{-1}$ and 1737 cm$^{-1}$, respectively. In the case of ascorbyl palmitate, two overlapping carbonyl peaks were identified between 1715 cm$^{-1}$ and 1800 cm$^{-1}$.

5.2.8. Quantification of ascorbyl palmitate

The amount of ascorbyl palmitate in the samples was estimated using a Bruker IFS 66/S FTIR Spectrometer (Bruker Optics, Billerica, MA) and $^1$H NMR as described in sections 4.2.5 and 4.2.6, respectively.

5.3. Results and Discussions

5.3.1. Formation of Hylon VII spherulites in the presence of fatty acid esters

Various processing conditions were used to evaluate the ability of Hylon VII starch to form spherulites in the presence of ascorbyl palmitate, retinyl palmitate and phytosterol esters. Processing conditions were selected based on previous studies on formation of starch spherulites. Round spherulites have been observed when a starch aqueous dispersion is heated to 180 °C followed by rapid cooling (Bhosale and Ziegler 2009; Creek and others 2006; Nordmark and Ziegler 2002a, 2002b; Ziegler and others 2005; Ziegler and others 2003). Cooling below 70 °C is necessary for the formation of this type of spherulite (Creek and others 2006). Spherulitic crystallization of starch induced by starch-fatty acid inclusion complexes has been observed when heating a starch aqueous dispersion in the presence of fatty acids to 140 °C, followed by rapid cooling to 90 °C, and then by slow cooling to 40 °C (Fanta and others 2008). Heinemann and others (2003; 2005) induced spherulitic crystallization by heating the starch dispersion to 120 °C followed by slow cooling to 30 °C, the temperature at which flavor compounds were added.
Peterson and others (2005) reported that spherulites that crystallize from starch inclusion complexes start forming around 84–86 °C, and Bhosale and Ziegler (2009) reported 82 °C as the temperature at which spherulites start to form. Therefore, in the present study, fatty acid esters were added in the starch dispersion at 90 °C during cooling, before spherulitic crystallization occurs. Fatty acid esters were not added before the heating process to avoid compound degradation caused by the high temperatures.

*Spherulite formation and morphology*

As shown in Figure 5.2, Figure 5.3, Figure 5.4, and Figure 5.5, a variety of morphologies were observed depending on the fatty acid ester and processing conditions used. Heating Hylon VII dispersion to 180 °C followed by rapid cooling resulted in the formation of round spherulites displaying the characteristic Maltese cross when viewed between cross polarizers (Figure 5.2). These observations are in agreement with previous studies on spherulite formation produced by heating Hylon VII starch dispersion to 180 °C followed by rapid cooling (Nordmark and Ziegler 2002a, 2002b; Suwanayuen 2009; Ziegler and others 2003). Similar to previous reports (Creek 2007; Creek and others 2006; Suwanayuen 2009; Ziegler and others 2005), Hylon VII control spherulites (without fatty acid ester addition) exhibited a B-type X-ray diffraction pattern (Table 5.3). Under the same processing conditions (heat to 180 °C followed by rapid cooling), Hylon VII did not form spherulites in the presence of ascorbyl palmitate. Instead, small particles with little or no birefringence were observed (Figure 5.3a). This sample exhibited a V-type X-ray diffraction pattern indicating the formation of starch inclusion complexes (Table 5.3). Based on FTIR analysis, ascorbyl palmitate remained in the sample after ethanol washing (Table 5.3), which suggests that this compound was forming inclusion complexes with the starch.
Figure 5.2. Hylon VII control samples (no fatty acid ester added, only presence of native lipids) observed under brightfield (left) and polarized (right) illumination, prepared by the following processes: a) heat to 180 °C, quickly cool to 120 °C, and cool to 40 °C at 1 °C/min; b) heat to 140 °C, quickly cool to 120 °C, and cool to 40 °C at 0.1 °C/min; and c) heat to 140 °C, quickly cool to 120 °C, and cool to 40 °C at 0.04 °C/min.
Figure 5.3. Hylon VII-ascorbyl palmitate samples observed under brightfield (left) and polarized (right) illumination, prepared by the following processes: a) heat to 180 °C, quickly cool to 120 °C, and cool to 40 °C at 1 °C/min; b) heat to 140 °C, quickly cool to 120 °C, and cool to 40 °C at 0.1 °C/min; and c) heat to 140 °C, quickly cool to 120 °C, and cool to 40 °C at 0.04 °C/min.
Figure 5.4. Hylon VII-retinyl palmitate samples observed under brightfield (left) and polarized (right) illumination, prepared by the following processes: a) heat to 180 °C, quickly cool to 120 °C, and cool to 40 °C at 1 °C/min; b) heat to 140 °C, quickly cool to 120 °C, and cool to 40 °C at 0.1 °C/min; and c) heat to 140 °C, quickly cool to 120 °C, and cool to 40 °C at 0.04 °C/min. Black arrows show “balloon” morphology.
Figure 5.5. Hylon VII-phytosterol ester samples observed under brightfield (left) and polarized (right) illumination, prepared by the following processes: a) heat to 180 °C, quickly cool to 120 °C, and cool to 40 °C at 1 °C/min; b) heat to 140 °C, quickly cool to 120 °C, and cool to 40 °C at 0.1 °C/min; and c) heat to 140 °C, quickly cool to 120 °C, and cool to 40 °C at 0.04 °C/min. Black arrows show “balloon” morphology.
Table 5.3. Thermal analysis, crystal type, and presence of fatty acid ester of Hylon VII-fatty acid ester samples

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<th>Fatty acid ester</th>
<th>T&lt;sub&gt;heat&lt;/sub&gt; (°C)</th>
<th>T&lt;sub&gt;int&lt;/sub&gt; (°C)</th>
<th>T&lt;sub&gt;cool&lt;/sub&gt; (°C)</th>
<th>Cooling rate (°C/min)</th>
<th>T&lt;sub&gt;p&lt;sub&gt;1&lt;/sub&gt;&lt;/sub&gt; (°C)</th>
<th>ΔH&lt;sub&gt;1&lt;/sub&gt; (J/g)</th>
<th>T&lt;sub&gt;p&lt;sub&gt;2&lt;/sub&gt;&lt;/sub&gt; (°C)</th>
<th>ΔH&lt;sub&gt;2&lt;/sub&gt; (J/g)</th>
<th>T&lt;sub&gt;p&lt;sub&gt;3&lt;/sub&gt;&lt;/sub&gt; (°C)</th>
<th>ΔH&lt;sub&gt;3&lt;/sub&gt; (J/g)</th>
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<th>Presence of fatty acid ester</th>
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<td>101.8</td>
<td>2.6</td>
<td>148.5</td>
<td>1.2</td>
<td>151.3</td>
<td>1.5</td>
<td>very weak B + V</td>
<td>No</td>
</tr>
<tr>
<td>Phytosterol ester</td>
<td>180</td>
<td>120</td>
<td>40</td>
<td>1</td>
<td>100.2</td>
<td>11.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>weak B and V</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>140</td>
<td>120</td>
<td>40</td>
<td>0.1</td>
<td>68.7</td>
<td>1.9</td>
<td>120.2</td>
<td>5.1</td>
<td>140.3</td>
<td>3.0</td>
<td>very weak B and V</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>140</td>
<td>120</td>
<td>40</td>
<td>0.04</td>
<td>82.2</td>
<td>9.3</td>
<td>151.3</td>
<td>1.5</td>
<td>150.8</td>
<td>1.7</td>
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<td>No</td>
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<td>120</td>
<td>40</td>
<td>1</td>
<td>99.7</td>
<td>11.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>B</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>140</td>
<td>120</td>
<td>40</td>
<td>0.1</td>
<td>76.4</td>
<td>4.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>amorphous</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>140</td>
<td>120</td>
<td>40</td>
<td>0.04</td>
<td>76.3</td>
<td>10.2</td>
<td>123.9</td>
<td>0.2</td>
<td>150.8</td>
<td>1.7</td>
<td>very weak B + V</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA = Not applicable
Tp = Peak temperature. ΔH = Enthalpy of endothermic transition
Creek and others (2007) reported that the presence of excess L-\(\alpha\)-lysophosphatidylcholine inhibited spherulitic crystallization. However, at very low concentration of this compound, spherulites were formed. On the other hand, Bhosale and Ziegler (2009) showed that these type of round spherulites can be formed in the presence of palmitic acid. In the present study, only ascorbyl palmitate prevented the formation of these round spherulites.

Heating Hylon VII dispersion to 180 °C followed by addition of retinyl palmitate or phytosterol ester at 90 °C during rapid cooling resulted in the formation of round spherulites (Figure 5.4a and Figure 5.5a) similar to those observed when Hylon VII starch was processed using the same heating treatment, but without fatty acid ester addition (Figure 5.2a). Spherulites formed in the presence of retinyl palmitate exhibited a very weak B-type pattern (Table 5.3). However, the X-ray diffraction pattern of round spherulites formed in the presence of phytosterol ester showed a mixture of B and V-type crystallinity indicating the presence of starch inclusion complexes. The idea of the presence of starch-phytosterol ester inclusion complexes was discarded because FTIR analysis showed that phytosterol esters were not present in this sample (Table 5.3). It is possible that starch inclusion complexes were formed with native lipids or some free fatty acids present in the commercial phytosterol esters used in the present investigation. It is possible that the lower water solubility of retinyl palmitate and phytosterol ester resulted in a lower amount of these molecules available in solution to form inclusion complexes with the starch and prevent spherulitic crystallization of these B-type round particles.

Small torus-shape birefringent particles were the main structures produced when heating Hylon VII control samples to 140 °C followed by slow cooling (Figure 5.2b and c). Few large spherical lobed particles with ringed birefringence (Figure 5.2b) could also be observed. Both type of structures have been previously reported when processing native common corn starch (Fanta and others 2005; Peterson and others 2005) and native high amylose maize starch (Fanta and others 2008) by steam jet cooking. Fanta and others (2006) explained that spherulite morphology depends on the fatty acid structure.
and other factors that can affect the rate of lamella formation and spherulitic growth, such as fatty acid solubility, cooling rate, and the ratio fatty acid:starch. Some large “balloon” like structures were also observed (Figure 5.2b) and will be discussed in more detail later.

In Hylon VII-ascorbyl palmitate samples heated to 140 °C followed by slow cooling, the torus-shape birefringent particles were the main structures formed, although some non-spherulitic material could be observed in the background of the images (Figure 5.3b and c). Torus-shape spherulites between 5–15 μm in diameter were produced at cooling rates of 0.1 °C/min. In addition to these particles, torus-shape spherulites with 10–20 μm diameter were also observed when cooling at 0.04 °C/min. In Hylon VII-ascorbyl palmitate samples heated to 180 °C and cooled at 1 °C/min, the faster cooling rate may not have allowed enough time for the inclusion complexes to organize in a spherulitic structure. In samples cooled at 0.04 °C/min, spherulites where large enough to distinguish the Maltese cross extinction pattern of those spherulites that were lying flat on the microscope slide (Figure 5.3c right). The Maltese cross extinction pattern is an indication of the radial organization of these structures.

Structures with various morphologies mixed in a gel-like material were observed in Hylon VII-retinyl palmitate and Hylon VII-phytosterol ester samples heated to 140 °C and cooled at 0.1 °C/min or 0.04 °C/min (Figure 5.4b-c and Figure 5.5b-c). These structures included 20–40 μm non-birefringent oval and irregular particles, various sizes of non-birefringent “balloon” morphologies, a few birefringent “torus-shape” particles, and gel-like material. Some rod-like birefringent morphologies could also be observed. In diluted samples, the rod-like particles tended to constantly flip showing that they were the side view of the torus-shape particles.

A “balloon” morphology has been previously reported when processing starch in the presence of certain molecules that forms starch inclusion complexes (Creek and others 2007; Egermayer and Piculell 2003). Creek and others (2007) observed a “balloon” structure when samples with more than 80% amylopectin were heated in the
presence of L-α-lysophosphatidylcholine. These authors suggested that these “balloon” morphologies could not be remnants of the original granular structure because they formed even when the starting material was non-granular. Egermayer and Piculell (2003) observed a “balloon” morphology when adding sodium dodecyl sulfate to an amylose solution heated to 155 °C and cooled to 90 °C. These authors referred these morphologies as unilamellar vesicles and suggested that they were formed by amylose inclusion complexes packed forming a lamella. Given the variety of structures and the gel-like material, it is not surprising the combined B and V-type X-ray diffraction pattern of these samples (Table 5.3).

Thermal analysis

Thermal analysis showed a main endothermic transition in all samples. The temperature of this transition varied depending on the fatty acid ester used and the temperature treatment (Table 5.3). All ascorbyl palmitate-Hylon VII samples showed the endothermic transition between 99 °C and 100 °C, which is around the dissolution temperature of amylose-ascorbil palmitate inclusion complexes (see section 4.3.1). In addition, in all these samples, FTIR analysis confirmed the presence of ascorbyl palmitate, which would have been removed during ethanol washing if the fatty acid ester was not included in a helical complex. Hence, the observed endothermic transition of these samples can be attributed to the dissolution of the observed particles formed by starch-ascorbil palmitate inclusion complexes. Previous studies reported a higher dissolution temperature (115–120 °C) for similar spherulites made from amylose-palmitic acid dispersions (Bhosale and Ziegler 2009), and from high amylose or normal corn starch dispersions (Fanta and others 2002), which were attributed to the dissolution of type II amylose-fatty acid complexes. However, as explained in section 4.3.1, amylose-ascorbil palmitate inclusion complexes have lower thermal stability than amylose-palmitic acid complexes.
For retinyl palmitate-Hylon VII samples heated to 180 °C, the main endothermic transition was between 70–130 °C with peak temperature at 99 °C. Hylon VII control spherulites formed under the same processing conditions also showed an endothermic transition within that temperature range (Table 5.3). This temperature is in agreement with the 90–100 °C melting temperature of high amylomaize starch spherulites reported by Nordmark and Ziegler (2002a). Because only B-type crystallinity was detected in retinyl palmitate-Hylon VII samples heated to 180 °C, the transition observed in these samples can be attributed to the dissolution of the round spherulites.

For phytosterol-Hylon VII samples heated to 180 °C, the main endothermic transition was broad between 60–135 °C with a peak temperature at 100 °C. X-ray diffraction pattern of this sample showed a mixture of B- and V-type crystallinity. As described above, the V-type crystallinity was likely caused by starch inclusion complexes with some free fatty acids present in the sample, possibly from the commercial phytosterol esters. As described by the manufacturer, phytosterol esters were made from canola oil fatty acids which are composed mainly by oleic, linoleic, and linolenic acids (Warner and others 1994). Starch inclusion complexes with these unsaturated fatty acids dissociate between 87–112 °C depending on the degree of unsaturation (Karkalas and others 1995; Lalush and others 2005; Tufvesson and others 2003), temperature that overlaps with the dissolution temperature range of these type of spherulites. Therefore, the main transition observed in these samples was likely due to both the melting of the spherulites and starch inclusion complexes with fatty acids.

Hylon VII controls heated to 140 °C and slowly cooled at 0.1 °C /min and 0.04 °C/min showed a broad and flat endotherm between 53–110 °C and 50–115 °C, respectively, with a peak temperature around 76 °C. These transition temperatures are lower than those previously reported by Fanta and others (2002) for Hylon VII spherulites produced by jet-cooking a dilute starch dispersion to 140 °C and slowly cooled at 0.04 °C/min. These authors reported the formation of the small torus-shape spherulites with a dissolution temperature of 115 °C, and large spherulites with an
additional endotherm around 90–105 °C. It is possible that the broad endotherm observed in Hylon VII controls heated to 140 °C was a combination of endothermic transitions, including the dissolution of the torus-shape spherulites, large spherulites, and possibly retrograded amylopectin, that overlapped to form a broad endotherm. The endotherm observed above 140 °C is most likely due to retrograded amylose. Both retrograded amylose and amylopectin were most likely the structures that produced the observed B-type X-ray diffraction pattern (Table 5.3).

Hylon VII-retinyl palmitate samples heated to 140 °C and cooled at 0.1 °C /min and 0.04 °C/min displayed two endothermic transitions. The main transition was around 96 °C and 102 °C for samples cooled at 0.1 °C/min and 0.04 °C/min, respectively. This main transition can be attributed to the dissolution of inclusion complexes displaying the V-type X-ray diffraction pattern. The second transition was at or above 140 °C, which can be attributed to retrograded amylose with B-type crystallinity. As reported in Section 4.3.2., the dissolution temperature of inclusion complexes of Hylon VII-retinyl palmitate-native lipids and Hylon VII-native lipids was 102 °C and 104 °C, respectively. However, based on FTIR analysis, Hylon VII-retinyl palmitate samples heated to 140 °C followed by slow cooling did not contain retinyl palmitate. Therefore, the V-type crystalline structures in these samples were likely formed between the starch molecules and native lipids.

Similarly, in Hylon VII-phytosterol samples heated to 140 °C and slowly cooled at 0.1 °C/min and 0.04 °C/min, the presence of phytosterol esters was not detected by FTIR analysis. Therefore, the endothermic transitions observed in these samples cannot be attributed to starch-phytosterol ester inclusion complexes. In samples cooled at a rate of 0.1 °C/min, a small endotherm around 69 °C was observed. In some of the measurements, thermograms also showed two additional endotherms at 120 °C and at 140 °C. The transition at 140 °C can be attributed to retrograded amylose with B-type crystallinity, while the transition at 120 °C is most likely due to the dissolution of type II amylose-native lipids inclusion complexes, possibly those forming the torus-shape
spherulites. The low temperature endotherm was in the range between 51 °C and 85 °C. This endotherm could represent the dissolution of retrograded amylopectin, and/or type I inclusion complexes with some free unsaturated fatty acids from the phytosterol ester. In samples cooled at a rate of 0.04 °C/min, the main endotherm ranged between 55 °C and 125 °C, with peak temperature around 82 °C. This endotherm probably represent the dissolution of the variety of observed structures such as the smaller torus-shape spherulites, retrograded amylopectin, and some type I and II inclusion complexes. The high temperature endotherm attributed to retrograded amylose was also observed.

The absence of retinyl palmitate and phytosterol ester in the final samples was likely caused by the limited solubility of these compounds in the reaction medium. On the other hand, the higher solubility of ascorbyl palmitate allowed these molecules to be available in solution to interact with the starch molecules and form inclusion complexes. Because only ascorbyl palmitate was entrapped in the starch precipitates after ethanol washing (Table 5.3), this compound was the only fatty acid ester that was studied further. The effect of processing parameters on spherulite formation in the presence of ascorbyl palmitate and efficiency of encapsulation measured as % entrapped ascorbyl palmitate in the dry samples were analyzed using a fractional factorial experiment. The total yield, % entrapped ascorbyl palmitate, and dissolution enthalpies from the main endothermic transition were analyzed.

5.3.2. Effect of processing parameters on spherulite formation and entrapment of ascorbyl palmitate

Crystallization of round spherulites have been observed at cooling rates of 1°C/min (Bhosale and Ziegler 2009) or faster (Creek 2007; Creek and others 2006; Nordmark and Ziegler 2002a, 2002b; Ziegler and others 2005; Ziegler and others 2003). On the other hand, for the formation of spherulites from inclusion complexes, very slow cooling rates, such as 0.04 °C/min, are necessary (Bhosale and Ziegler 2009; Fanta and others 2008). However, at intermediate cooling rates around 0.5 °C/min, spherulitic
crystallization has not been observed (Bhosale and Ziegler 2009). Thus, a logarithmic transformation of the cooling rate was used to ensure that the center point of the design did not lie at a cooling rate at which spherulites are not formed. Therefore the minimum and maximum levels used for cooling rate were Log(0.04) = -1.4 and Log (1) = 0, with center point at -0.7 which is the Log(0.2).

The intermediate temperature was set constant at 90 °C and not at 120 °C to avoid confounding effects between the temperature at which the sample was quickly cooled, and the temperature at which the sample was held after addition of ascorbyl palmitate. Previous studies have shown spherulitic crystallization of starch when heating the starch aqueous dispersion to 140 °C (Bhosale and Ziegler 2009; Fanta and others 2008) or 180 °C (Bhosale and Ziegler 2009) followed by rapid cooling to 90 °C.

Depending on the processing conditions, various types of structures were produced (Figure 5.6), including round spherulites, torus-shape spherulites of various sizes, non spherulitic birefringent or non birefringent particles, “balloon” morphologies of around 20 μm in size, and gel. Small torus shape spherulites were less than 5 μm in diameter with a height of 1–2 μm, medium size torus shape spherulites ranged from 5 μm to 10 μm in diameter with a height of 2–4 μm, and large torus shape spherulites ranged from 10 μm to 20 μm in diameter with a height of 4–9 μm. It is possible that the observed “balloon” structures are similar to the unilamellar vesicles reported by Egermayer and Piculell (2003). These authors suggested that these vesicles are surrounded by a single lamella composed by a layer of amylose helices forming inclusion complexes. In most of the treatments, a mixture of morphologies was obtained which will be discussed in more detail later.

The % yield increased at higher concentrations of Hylon VII (Table 5.4 and Figure 5.7a). With the exception of experiment 1, treatments that had the highest yields (above 75%), were produced with 8% Hylon VII heated to 140 °C, and produced a large
Figure 5.6. Morphology of Hylon VII-ascorbyl palmitate complexes observed under brightfield (left) and polarized (right) illumination: a) Mixture of various structures. Black arrows show the “balloon” morphology (experiment 1 large fraction), b) Round spherulites (experiment 14 large fraction), c) large torus-shape spherulites (experiment 4 large fraction), d and e) medium size torus-shape spherulites (experiment 12 and 16), f) small torus-shape spherulites (experiment 14 small fraction), g) non spherulitic and non birefringent particles (experiment 11 small fraction), and h) non spherulitic birefringent particles (experiment 13 small fraction)
Figure 5.6. (continued)
proportion of gel (see experiments 2, 4, 10, and 12 in Table 5.5). Based on the V-type X-ray diffraction pattern observed in all treatments, inclusion complexes were formed in all samples (Table 5.5). In these treatments (8% Hylon VII and heated to 140 °C), starch molecules may interact among each other forming a network resulting in a large proportion of gel-like material. On the other hand, amylose solutions with less than 1.5% amylose concentration do not form a gel (Eerlingen and Delcour 1995). Hence, in treatments with low starch concentration (2%), the starch solution may be too diluted to form a gel. Thus, it is likely that this significant increased in % yield with higher % Hylon VII was caused by the formation of the gel-like material.

An inverse relationship was observed between % yield and $T_{\text{heat}}$ (Table 5.4 and Figure 5.7b). Creek and others (2006) proposed that when heating amylose between 130
°C and 160 °C in the presence of excess water, double helical structures and fixed network entanglements dissociate leaving regions of rigid helical conformation that act as helical nuclei promoting gel formation on cooling. In the present study, these helical nuclei possibly remained in treatments heated to 140 °C, favoring gel formation resulting in higher yield. The number and length of these helical nuclei decreases with further heating, and they dissociate between 160 °C and 180 °C (Creek and others 2006). An inverse relationship was also observed between % yield and cooling rate (Table 5.4 and Figure 5.7c). At slower cooling rates, starch molecules have longer time for complexation and network formation, resulting in higher yield.

Table 5.4. Analysis of variance and regression coefficients for the effect of % Hylon VII (g dry Hylon VII/100 g dispersion), % added ascorbyl palmitate (g ascorbyl palmitate/100 g amylose), Theat, holding time at the Tint of 90 °C, cooling rate, and Tcool on % yield, % entrapped ascorbyl palmitate, and dissolution enthalpy.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>% yield</th>
<th>% entrapped ascorbyl palmitate</th>
<th>ΔH1 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>df</td>
<td>Sum of Squares 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>10177</td>
<td>57</td>
</tr>
<tr>
<td>Regression</td>
<td>8</td>
<td>9386*</td>
<td>43*</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>791</td>
<td>14</td>
</tr>
<tr>
<td>Lack of fit</td>
<td>8</td>
<td>787*</td>
<td>13</td>
</tr>
<tr>
<td>Pure error</td>
<td>2</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

R2 = 0.92, 0.76, 0.85

<table>
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<tr>
<th>Parameter</th>
<th>Regression coefficients 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>163.3</td>
</tr>
<tr>
<td>% Hylon VII</td>
<td>7.5*</td>
</tr>
<tr>
<td>% added ascorbyl palmitate</td>
<td>-0.4</td>
</tr>
<tr>
<td>Theat</td>
<td>-0.9*</td>
</tr>
<tr>
<td>Holding time</td>
<td>-0.1</td>
</tr>
<tr>
<td>Log(cooling rate)</td>
<td>-30.3*</td>
</tr>
<tr>
<td>Tcool</td>
<td>-0.2</td>
</tr>
<tr>
<td>% Hylon VII*% added ascorbyl palmitate</td>
<td>-0.2</td>
</tr>
<tr>
<td>% Hylon VII*Log(cooling rate)</td>
<td>4.1*</td>
</tr>
</tbody>
</table>

1 Experiment 19 produced insufficient amount of sample for thermal analysis. Therefore, experiment 19 was excluded for the statistical analysis and dftotal = 17, dfrresidual = 9, and dfLack of fit = 7.

2 Sum of squares or regression coefficients followed by * show statistical significance at α=0.05.
The interaction effect between % Hylon VII and cooling rate on % yield was significant (Table 5.4). At 2% starch concentration, the %yield increased when the cooling rate was decreased (Figure 5.8). However, at 8% starch concentration, cooling rate did not significantly affect the % yield. Cooling quickly a low starch concentration dispersion may not allow enough time for starch and guest molecules to diffuse closer to each other and form inclusion complexes or a starch gel. At a slower cooling rate, molecules in the 2% starch dispersion will have more time for diffusion, complexation, and gel formation. On the other hand, at higher starch concentration, starch molecules will need less time to diffuse towards guest or other starch molecules to form complexes or a network, and cooling at 1 °C/min may allow sufficient time for these phenomena to occur.

The regression model for % yield showed a significant lack of fit (Table 5.4). It is possible that this lack of fit was caused by a discontinuity in spherulitic formation within the range of heating temperatures used. For example, round spherulites are not formed if
Table 5.5. Light microscopy evaluation, yield, %ascorbyl palmitate, thermal analysis, and crystal type of Hylon VII-ascorbyl palmitate fractions from fractional factorial experiment

<table>
<thead>
<tr>
<th>Exp No</th>
<th>Run No</th>
<th>Fraction</th>
<th>Observations¹</th>
<th>Yield (%)</th>
<th>% entrapped Asc.Palm.²</th>
<th>Tp1 (°C)</th>
<th>ΔH1 (J/g)</th>
<th>Tp2 (°C)</th>
<th>ΔH2 (J/g)</th>
<th>Crystal type</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>1</td>
<td>Small</td>
<td>Mostly small torus</td>
<td>75.6</td>
<td>0.10</td>
<td>83.3</td>
<td>3.6</td>
<td>139.8</td>
<td>1.9</td>
<td>Very weak V</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large</td>
<td>Mostly large torus + few small and medium torus + balloons</td>
<td>22.4</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>Small</td>
<td>Medium torus + small non-spherulitic particles</td>
<td>13.8</td>
<td>0.65</td>
<td>83.4</td>
<td>3.9</td>
<td>141.7</td>
<td>1.7</td>
<td>Weak V</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medium</td>
<td>Gel with medium and large torus</td>
<td>25.8</td>
<td>0.70</td>
<td>95.2</td>
<td>0.9</td>
<td>143.1</td>
<td>0.8</td>
<td>Weak V + weak B</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large</td>
<td>Gel + large torus</td>
<td>54.5</td>
<td>0.49</td>
<td>93.8</td>
<td>1.2</td>
<td>145.7</td>
<td>1.4</td>
<td>Weak V + weak B</td>
</tr>
<tr>
<td>3</td>
<td>17</td>
<td>No fractionation</td>
<td>Mostly non spherulitic particles + balloons + few medium torus</td>
<td>42.2</td>
<td>3.94</td>
<td>103.3</td>
<td>3.0</td>
<td>127.8</td>
<td>1.0</td>
<td>Very weak V</td>
</tr>
<tr>
<td>4</td>
<td>14</td>
<td>Small</td>
<td>Medium torus + non spherulitic particles</td>
<td>18.0</td>
<td>3.71</td>
<td>100.8</td>
<td>3.6</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medium</td>
<td>Various size of torus</td>
<td>9.2</td>
<td>3.68</td>
<td>101.1</td>
<td>4.3</td>
<td>nd</td>
<td>nd</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large</td>
<td>Mixture of various sizes embedded in gel</td>
<td>51.0</td>
<td>0.52</td>
<td>79.6</td>
<td>3.6</td>
<td>155.4</td>
<td>0.9</td>
<td>Weak V + weak B</td>
</tr>
<tr>
<td>5</td>
<td>19</td>
<td>No fractionation</td>
<td>Small torus + non spherulitic particles + gel</td>
<td>15.4</td>
<td>0.00</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>Small</td>
<td>Medium torus</td>
<td>20.8</td>
<td>0.51</td>
<td>99.1</td>
<td>0.5</td>
<td>nd</td>
<td>nd</td>
<td>Weak V</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large</td>
<td>Large round spherulites</td>
<td>35.9</td>
<td>0.00</td>
<td>97.2</td>
<td>12.3</td>
<td>nd</td>
<td>nd</td>
<td>Very weak V + very weak B</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>No fractionation</td>
<td>Medium torus + some non spherulitic particles and sheets + few round spherulites</td>
<td>40.9</td>
<td>1.45</td>
<td>97.4</td>
<td>6.4</td>
<td>nd</td>
<td>nd</td>
<td>Very weak V</td>
</tr>
<tr>
<td>8</td>
<td>18</td>
<td>Small</td>
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<td>3.38</td>
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¹ Very small torus <1 μm, small size torus 2–10 μm, and large torus 10–20 μm in diameter
² % Asc.Palm = % ascorbyl palmitate in dry sample (g ascorbyl palmitate/100 g starch)
nd = not determined due to insufficient sample
Table 5.5. (continued)

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<th>Exp No</th>
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<th>Fraction</th>
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<th>Yield (%)</th>
<th>% entrap Asc. Palm.(^2)</th>
<th>Tp(_1) (°C)</th>
<th>ΔH(_1) (J/g)</th>
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<td></td>
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<td>Large</td>
<td>Gel + various size of torus + balloons</td>
<td>77.1</td>
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<td></td>
<td></td>
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<td>Mix various size torus + balloons</td>
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<td>Various size of torus</td>
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<td>100.8</td>
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<tr>
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<td></td>
<td>Large</td>
<td>Gel + various size of torus</td>
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<td>0.77</td>
<td>75.4</td>
<td>5.0</td>
<td>146.4</td>
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<td>Weak V</td>
</tr>
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<td>13</td>
<td>9</td>
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<td>Non spherulitic particles</td>
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<td>nd</td>
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<td>3.7</td>
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<td>nd</td>
<td>97.8</td>
<td>4.9</td>
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<td></td>
<td>Very weak V</td>
</tr>
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<td>Round spherulites + few medium torus + little gel</td>
<td>3.7</td>
<td>0.93</td>
<td>101.3</td>
<td>1.6</td>
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<td>Weak V</td>
</tr>
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<td>Medium torus</td>
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<td></td>
<td>V</td>
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<td>6.4</td>
<td>3.37</td>
<td>106.3</td>
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<td></td>
<td>Weak V</td>
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</tr>
<tr>
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<td>Medium torus + birefringent particles</td>
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<td>nd</td>
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<tr>
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<td>50.4</td>
<td>1.38</td>
<td>98.8</td>
<td>3.9</td>
<td></td>
<td></td>
<td>Weak V</td>
</tr>
</tbody>
</table>

\(^1\) Small torus <5 μm, medium torus 5–10 μm, and large torus 10–20 μm in diameter
\(^2\) % Asc.Palm = % ascorbyl palmitate in dry sample (g ascorbyl palmitate/100 g starch)
nd = not determined due to insufficient sample
the starch dispersion is heated below 164 °C (Ziegler, personal communication, 2009). It is also possible that the variation of % yield of the replicates at the center point was very low compared to the variability of the other treatments, and thus, the pure error was not representative of the true experimental error.

Figure 5.8. Interaction effect of % Hylon VII and cooling rate on % yield of precipitates. The remaining factors were kept constant at the center level. Predicted values are shown. Error bars represent 95% confidence interval.

The % entrapped ascorbyl palmitate was significantly affected by the % added ascorbyl palmitate (Table 5.4 and Figure 5.9). At higher concentrations of added ascorbyl palmitate, more guest molecules will be available in the dispersion to interact with the starch resulting in higher complexation. Interestingly, cooling rate did not significantly affect % entrapped ascorbyl palmitate. A faster cooling rate would allow less time for complex formation. However, it is possible that even the fastest cooling rate (1 °C/min) provided enough time for complexation of all available ascorbyl palmitate. It is also possible that the effect of cooling rate on % entrapment may have been counteracted by other phenomena occurring at the same time. For example, the slower
the cooling rate, the longer time ascorbyl palmitate remains at higher temperatures, which can result in higher ascorbyl palmitate degradation. In addition, as described above, slower cooling rates also allow more time for the formation of a starch gel. Based on the presence of an endotherm above 140 °C and the B-type X-ray diffraction pattern of fractions with gel-like material, the gel contained retrograded amylose that do not form inclusion complexes (Karkalas and others 1995), and therefore the gel entraps less proportion of ascorbyl palmitate.

Figure 5.9. Effect of % added ascorbyl palmitate (w/w Hylon VII) on % entrapped ascorbyl palmitate (w/w Hylon VII) in the precipitate. The remaining factors were kept constant at the center level. (—) shows the predicted line and error bars represent 95% confidence interval.

The interaction effect between % Hylon VII and % added ascorbyl palmitate on % entrapped ascorbyl palmitate was significant (Table 5.4). The increasing effect of % added ascorbyl palmitate on % entrapment was more prominent at lower starch concentration (Figure 5.10). As explained above, the higher the % added ascorbyl palmitate, the more guest molecules will be available in the dispersion to form starch inclusion complexes increasing the % entrapment. However, at 8% starch concentration, in addition to inclusion complex formation, starch molecules retrograde forming a gel,
which may contain little or no ascorbyl palmitate, resulting in a lower % entrapment in the precipitate.

Figure 5.10. Interaction effect of % Hylom VII and % added ascorbyl palmitate on % entrapped ascorbyl palmitate in the precipitates. The remaining factors were kept constant at the center level. Predicted values are shown. Error bars represent 95% confidence interval.

A main endothermic transition was observed in all treatments. In most cases, the peak temperature of this transition (T\textsubscript{p1}) was between 93 °C to 103 °C, but it could range from 60 °C to 110 °C (Table 5.5). This endothermic transition most likely represents the dissolution of the various structures formed, including round spherulites, torus-shape spherulites, and non-spherulitic inclusion complexes. Figure 5.11 shows DSC thermograms of the various types of structures formed. The broad range of T\textsubscript{p1} is not surprising given the variety of structures observed. For example, as shown in Table 5.3, the dissolution of spherulites made by heating a starch dispersion to 140 °C without fatty acid ester addition was around 76 °C, while the dissolution of round spherulites formed by heating the starch dispersion to 180 °C was around 100 °C.
Figure 5.11. Representative DSC thermograms of a low temperature endotherm (experiment 1 small fraction), non spherulitic particles (experiment 11 small fraction), round spherulites (experiment 6 large fraction), small torus-shape spherulites (small fraction experiment 8), and a mixture of large and medium spherulites in gel (experiment 17 large fraction).

The enthalpy of the main endothermic transition ($\Delta H_1$) varied among treatments and ranged from 1.5 J/g to 10 J/g (Table 5.2). As shown in Figure 5.12, this enthalpy increased with higher $T_{\text{heat}}$ and decreased at faster cooling rates. The significant effect of $T_{\text{heat}}$ on $\Delta H_1$ was likely caused by the different type of structures formed at the studied heating temperatures. A large proportion of round spherulites and/or non-spherulitic particles were observed in treatments heated to 180 °C that had the highest $\Delta H_1$ (>6 J/g) (see experiments 6, 7, 13, 14, and 15 on Table 5.2 and Table 5.5). As shown in Table 5.5, $\Delta H_1$ of round spherulites was around 12 J/g (see experiment 6 and 14 on Table 5.5), and $\Delta H_1$ of some non-spherulitic particles was around 8 J/g (see experiment 13 on Table 5.5). On the other hand, in treatments heated to only 140 °C, as explained previously, helical nuclei may have remained which favored gel formation. As a result, more
precipitate (i.e. higher yield) but with lower proportion of inclusion complexes (then lower $\Delta H_1$) is formed at lower $T_{\text{heat}}$.

Figure 5.12. Effect (a) heating temperature and (b) cooling rate on the dissolution enthalpy $\Delta H_1$ (J/g). The remaining factors were kept constant at the center level. (—) shows the predicted line and error bars represent 95% confidence interval.

In addition to the differences in structures formed at the various heating temperatures studied, other phenomena occurring at high temperatures may have contributed to the higher $\Delta H_1$. Vesterinen and others (2001) proposed that at temperatures above 140 °C, amylopectin degrades increasing the ratio of linear polymers. Nordmark and Ziegler (2002b) also showed that only traces of amylopectin are present after processing high amylose starch using three heating cycles to 180 °C. Therefore, at higher heating temperatures, the degree of amylopectin degradation increases possibly increasing the amount of linear molecules, which are more prone to form inclusion complexes (Villwock and others 1999) and spherulites (Nordmark and Ziegler 2002a).

An inverse relationship was observed between $\Delta H_1$ and cooling rate (Table 5.4 and Figure 5.12b). A slower cooling rate would allow more time for complexation and spherulitic crystallization and therefore higher $\Delta H_1$. 
Even though V-type crystallinity was observed in all treatments (Table 5.5), in most cases, a weak or very weak diffraction pattern was obtained suggesting a low percentage of crystallinity of the samples. X-ray diffraction pattern of some of the treatments showed a mixture of V- and B-type crystallinity (Figure 5.13). The observed B-type crystallinity likely corresponded to the gel structure or round spherulites formed in those treatments (See experiments 2, 4, 6, 10, 12, and 14 on Table 5.5). As discussed earlier, previous studies have shown that these type of round spherulites have B-type crystallinity (Creek 2007; Nordmark and Ziegler 2002a; Suwanayuen 2009; Ziegler and others 2005). In those samples where the gel structure was formed, an endothermic transition above 140 °C was observed (See $T_{p_2}$ on Table 5.5) which is likely caused by the dissolution of amylose structures with B-type crystallinity forming the gel.

In addition to torus shape spherulites, a large proportion of round spherulites were observed in experiments 6 and 14. Bhosale and Ziegler (2009) also observed a mixture of two morphologies, round spherulites and non-spherulitic particles, when an amylose-palmitic acid aqueous dispersion was heated to 180 °C followed by cooling at 1 °C/min to 40 °C. These authors suggested that the two observed morphologies possibly had different type of crystallinity based on the mixed B- and V-type X-ray diffraction pattern of the sample.

The common processing parameters of experiments 6 and 14 were 8% Hylon VII, 2% added ascorbyl palmitate, $T_{\text{heat}} = 180$ °C, and cooling rate = 0.04 °C/min. Because these type of spherulites are less prone to form at very slow cooling rates (Creek and others 2006; Nordmark and Ziegler 2002a; Ziegler and others 2005), their formation was surprising given the 0.04 °C/min cooling rate of these treatments. In addition, as shown in Figure 5.3, round spherulites were not formed when cooling at 1 °C/min a starch dispersion heated to 180 °C in the presence of ascorbyl palmitate. These type of spherulites were not common in the other treatments where $T_{\text{heat}} = 180$ °C and cooling rate = 1 °C/min.
Creek and others (2006) reported that rapid cooling from 180 °C to 130 °C results in more spherulitic crystallization. These authors hypothesized that rapid cooling induces phase separation into a polymer rich and polymer poor phases, prior to network formation. Thus, in the present study, rapid cooling from 180 °C to 90 °C likely resulted in phase separation and consequently, a gel was not formed despite the slow cooling rate from 90 °C to the final temperature. Experiments 6 and 14 comprised the highest % Hylon VII and the lowest % added ascorbyl palmitate. It appears that under these conditions, starch-ascorbyl palmitate inclusion complexes are first formed inducing spherulitic crystallization of the smaller torus-shape structures, which occurs around 82–
84 °C (Bhosale and Ziegler 2009; Peterson and others 2005). Then, below 70 °C round spherulites are formed (Creek and others 2006) from the excess starch that did not form inclusion complexes. This is in agreement with the absence of ascorbyl palmitate in the round spherulites and entrapment of this compound in the torus-shape structures (See Table 5.5 experiments 6 and 14).

On the other hand, in experiments 2 and 10, a large proportion of gel was formed instead of the round spherulites, even though these treatments also comprised the highest % Hylon VII and lowest % added ascorbyl palmitate. These treatments were heated only to 140 °C. Creek and others (2006) hypothesized that at temperatures below 170 °C helical nuclei remain resulting in gel formation on cooling.

Throughout the fractional factorial experiment, torus-shape spherulites of various sizes were observed. Sizes ranged from less than 5 μm to almost 20 μm. In general, torus-shape spherulites larger than 10 μm were formed when the starch dispersion was heated to 140 °C. The only treatment heated to 140 °C that did not produce large torus-shape spherulites was experiment 3. In this treatment, the 2% starch dispersion was quickly cooled at 1 °C/min to 40 °C. The V-type X-ray diffraction pattern, the endothermic transition at 103 °C, and the 3.9% entrapped ascorbyl palmitate indicates that complex formation between starch and ascorbyl palmitate occurred in this treatment. However, the short treatment (50 minutes) may have not allowed enough time for spherulitic grow. Instead, a large proportion of non spherulitic particles were formed.

Variability in the FTIR spectra was observed between treatments and between fractions of the same treatments. Figure 5.14 shows representative FTIR spectra of various samples. As explained in Section 4.3.1, the carbonyl peaks in the FTIR spectrum of starch-ascorbil palmitate inclusion complexes (Figure 5.14 (vi)) slightly shifted to a higher wave number as compared to the spectra of the pure compounds (Figure 5.14 (viii)) and the physical mixture of Hylon VII starch and ascorbyl palmitate (Figure 5.14 (vii)). In some samples, the two characteristic carbonyl peaks were easily identified
(Figure 5.14 (ii) and (iv)) in particular after subtracting the starch spectrum. However, broadening of the peaks could be observed in some precipitates (Figure 5.14 (ii)). In some samples, a broad peak around 1740 cm\(^{-1}\) was observed instead of the two carbonyl peaks (Figure 5.14 (v)), similar to that observed for Hylon VII-ascorbyl palmitate physical mixtures at 0.5%. In other cases, the intensity of the carbonyl peak around 1740 cm\(^{-1}\) was much higher than expected (Figure 5.14 (iii)). The variability observed in the FTIR spectra among samples suggests that ascorbyl palmitate molecules may be interacting with the starch in different ways depending on the processing conditions and structure formed. It is also possible that under certain processing conditions ascorbyl palmitate molecules may be changing or degrading, resulting in a change in their spectrum.

5.4. Conclusions

Spherulitic crystallization in the presence of fatty acid esters depended on the fatty acid ester and thermal process used. It appears that the solubility of the fatty acid ester in the reaction medium plays a major role in the formation of spherulites. It is possible that low solubility fatty acid esters may not be available in solution to form enough inclusion complexes to prevent the formation of round spherulites, or induce the crystallization of spherulites from inclusion complexes. On the other hand, the higher solubility of ascorbyl palmitate results in higher starch complexation preventing round spherulite formation or inducing crystallization of torus-shape particles.

Morphology, yield, and amount of inclusion complexes in the precipitates appear to depend on the processing conditions, in particular the heating temperature and cooling rate. However, the amount of entrapped ascorbyl palmitate in the starch precipitate seems to be governed by the amount of this compound added during processing. This study shows that starch spherulites can be formed from starch-ascorbyl palmitate complexes, which suggests the potential use of starch spherulites for the encapsulation of esters of fatty acids with water soluble bioactive compounds. However, additional
research is needed to better understand spherulite formation and the type of molecules that can be entrapped in these spherulites.

Figure 5.14. FTIR spectra of (i) Hylon VII, (ii) experiment 11 small fraction (iii) experiment 11 large fraction, (iv) experiment 16 small fraction, (v) experiment 17 small fraction, (vi) amylose-ascorbyl palmitate inclusion complex, (vii) Hylon VII-ascorbyl palmitate physical mixture, and (viii) pure ascorbyl palmitate.
5.5. References


Fanta GF, Felker FC, Shogren RL, Salch JH. 2006. Effect of fatty acid structure on the morphology of spherulites formed from jet cooked mixtures of fatty acids and defatted corn starch. Carbohydrate Polymers 66(1):60-70.


Chapter 6

CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH

The results presented in this study showed that spherulitic crystallization of starch is greatly affected by the proportion and structure of the starch fractions, amylose, amylopectin, and the intermediate material. Higher ratios of linear to branched molecules resulted in the formation of larger amounts of rounder spherulites with higher dissolution temperature. In addition to the presence of branches, it appears that spherulitic crystallization is also affected by other factors, such as degree of branching, chain length, and chain length distribution. Because the ratio of amylose to amylopectin and the molecular structure of the starch fractions can affect the spherulitic crystallization of starch, these factors should be taken into account when selecting a starch source for spherulite formation.

Amylose and Hylon VII starch can form inclusion complexes with esters of fatty acids with ascorbic acid, retinol, or phytosterols. The presence of native lipids in Hylon VII starch did not inhibit complex formation. On the contrary, native lipids appear to increase the yield and thermal stability of the starch-fatty acid ester inclusion complexes, possibly due to the formation of ternary complexes.

Formation of starch inclusion complexes with fatty acid esters seem to be limited to the solubility of the guest compound in the reaction medium and the structure of the molecules forming the ester with the fatty acid: The smaller and more soluble the molecule forming the ester, the higher the complexation efficiency. The length of the helix of amylose-ascorbyl palmitate inclusion complexes is suggested to be affected by the amount of ascorbyl palmitate added during processing. At low concentrations of ascorbyl palmitate (<5% w added ascorbyl palmitate/w starch) shorter chains may be formed. At intermediate ascorbyl palmitate concentrations (10–20% w added ascorbyl
palmitate/w starch) a second population of longer helices may be formed, and at high concentration of ascorbyl palmitate (50% w added ascorbyl palmitate/w starch) only longer helices with higher thermal stability are produced. It is hypothesized that at low ascorbyl palmitate concentration, one ascorbyl palmitate molecule is included in the amylose helix. As the concentration of ascorbyl palmitate increased, two ascorbyl palmitate molecules, possibly arranged tail to tail, are located inside the helix, resulting in complexes with higher thermal stability, higher % crystallinity, and higher % entrapped ascorbyl palmitate.

The solubility of fatty acid esters appears to play a major role in their entrapment in starch spherulites. The higher water solubility of ascorbyl palmitate resulted in higher starch complexation inducing spherulitic crystallization of torus-shape particles. Morphology, yield, and amount of inclusion complexes in the precipitate appear to depend on processing conditions, in particular the heating temperature and cooling rate. However, the amount of entrapped ascorbyl palmitate in the starch precipitate seems to be governed by the amount of this compound added during processing.

In the study of the effect of processing parameters on spherulite formation and entrapment of ascorbyl palmitate, in addition to the formation of inclusion complexes, amylose also associated forming double helices producing round spherulites and/or gel, particularly at high starch concentration (8% w/w dispersion). The dissolution of round spherulites takes place within the same temperature range of inclusion complexes, which limits the use of the dissolution enthalpy ($\Delta H_1$) as a direct estimate of the amount of inclusion complexes present in the precipitate. The formation of gel restricted the separation of the different produced morphologies, limiting the evaluation of the effect of processing parameters on the formation of torus-shape spherulites. Thus, the concentration of starch in the dispersion should be reduced to decrease the formation of round spherulites and/or gel, which entrap little or no ascorbyl palmitate.
This study suggests the potential use of starch spherulites for the encapsulation of esters of fatty acids with water soluble bioactive compounds. However, further research is necessary to gain a better understanding of the type of molecules that can be entrapped in starch spherulites, and the factors affecting spherulitic crystallization and bioactive compound entrapment.

Suggestions for future research include to: a) study the stability of the entrapped fatty acid esters at various storage and processing conditions including time, temperature, and pH; b) study the release of the entrapped fatty acid esters from starch inclusion complexes and spherulites under stomach and small intestine conditions; c) to study systematically the effect of solubility and molecular structure of fatty acid esters on starch inclusion complex and spherulite formation, d) to optimize processing conditions in order to maximize the amount of formed inclusion complexes and spherulites containing the active compounds, and at the same time, maximize the amount of entrapped fatty acid esters.
Appendix A

CHAPTER 3 – SUPPLEMENTAL DATA

Figure A.1. SEM images of spherulites made from various proportions of Hylon VII amylose, intermediate material, and amylopectin. White arrows show what appears to be a central cavity.
Table B.1. Comparison of the estimated % ascorbyl palmitate in precipitates using FTIR and NMR

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<th>% ascorbyl palmitate in precipitate</th>
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</tbody>
</table>

Figure B.1. % ascorbyl palmitate in precipitates estimated using FTIR and NMR as a function of % added ascorbyl palmitate during processing.
Figure B.2. Comparison of the estimated % ascorbyl palmitate in precipitates using FTIR and NMR

\[ y = 1.8899x + 2.0999 \]

\[ R^2 = 0.8922 \]
Figure B.3. FTIR standard curves for the estimation of a) % ascorbyl palmitate, b) % retinyl palmitate, and c) % phytosterol ester in precipitates.
Figure B.3. (Continued)

Figure B.4. DSC curves of native Hylon VII and defatted Hylon VII controls (no fatty acid ester addition) for the formation of inclusion complexes in DMSO at 90 °C.
Figure B.5. Amount of fatty acid ester entrapped in precipitates of native Hylon VII with a) retinyl palmitate, or b) phytosterol ester. Error bars represent the standard deviation.
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