HIERARCHICAL STRUCTURE OF FIELD PEA STARCHES AND THEIR IMPACT ON PHYSICOCHEMICAL PROPERTIES

by

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Abstract

The objective of this study was to determine the molecular structure and properties of newly released cultivars of field peas [CDC Golden (CDCG), Abarth (ABAR), CDC Patrick (CDCP) and CDC Amarillo (CDCA)] grown at different locations in Saskatchewan, Canada. Starch yield (on a whole seed basis), apparent amylose, total lipid and surface area were in the range 34-37%, 38.2-42.6%, 1.07–1.38% and 0.31-0.38 m^2, respectively. The proportion of short (DP 6-12) amylopectin chains, amylopectin branching density, molecular order, crystallinity, crystalline heterogeneity, gelatinization transition temperatures, pasting temperatures, peak viscosity, extent of acid hydrolysis, and resistant starch content were higher in CDCG and ABAR. However, amylopectin long chains (DP 13-26), average chain length and thermal stability were higher in CDCP and CDCA. The results of this study showed that differences in physicochemical properties among cultivars were mainly influenced by amylopectin chain length distribution, amylopectin branching density and co-crystallization of amylose with amylopectin.

Keywords: field pea starch; structure; properties

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AAM	-	Apparent amylose content
ADP	-	Adenosine diphosphate
AFM	-	Atomic force microscopy
AM	-	Amylose
AMD	-	Arithmetic mean diameter
AML	-	Amylose leaching
AP	-	Amylopectin
APCLD	-	Amylopectin chain length distribution
ATP	-	Adenosine triphosphate
ATR-FTIR	-	Attenuated total reflectance Fourier transform Infrared spectroscopy
BV	-	Breakdown viscosity
^{13}C CP/MAS NMR	-	Cross-polarization magic angle spinning carbon-13 nuclear magnetic resonance
CDC	-	Crop Development Centre
$\overline{CL}$	-	Chain length
DMSO	-	Dimethyl sulphoxide
DP	-	Degree of polymerisation

DSC	-	Differential scanning calorimetry
FV	-	Final viscosity
GOPOD	-	Glucose oxidase/peroxidase
HPAEC-PAD	-	High performance anion exchange chromatography with pulsed amperometric detection
HPSEC	-	High performance size exclusion chromatography
LC	-	Long chain
RC	-	Relative crystallinity
RDS	-	Rapidly digestible starch
RS	-	Resistant starch
RVA	-	Rapid visco analyzer
SBV	-	Setback viscosity
SC	-	Short chain
SDS	-	Slowly digestible starch
SSA	-	Specific surface area
T_c	-	Conclusion temperature
T_o	-	Onset temperature
T_p	-	Peak temperature
UDP	-	Uridine diphosphate
WAXS	-	Wide angle X-ray scattering

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Chapter 1

Introduction and overview

Legumes are dicotyledenous seeds of plants belonging to the family Leguminosae (Allen & Allen, 1981). They are the third largest flowering plant family comprising 727 genera and 19325 species. Legumes, referred to as the poor man's meat play a prominent role in human nutrition as they are a good source of proteins, calories, minerals and vitamins (Deshpande, 1992). The presence of root and stem nodules containing nitrogen fixing bacteria is the characteristic feature of legumes. Owing to the development of chemical fertilizers and herbicides, there has been a drastic reduction in incorporating legumes for crop rotations (McCartney & Fraser, 2010). Grain legumes are cultivated in both tropical and temperate regions across the world (Iqbal, Khalil, Ateeq, & Khan, 2006) and they constitute 33% of the dietary protein needs of humans (Singh, Singh, Chung, & Nelson, 2007). Although grain legumes have been consumed for many centuries, only some years back their beneficial effects were investigated using suitable approaches (Duranti, 2006). Legumes are classified into two types: oilseeds that include soybeans and peanuts that are grown for their protein and oil content and grain legumes, comprising common beans, lentils, chickpeas and common peas that are grown for their protein (Venter & Van Eyssen, 2001). Starch, fibre and dietary fibre are the main components of grain legumes (Guillon & Champ, 2002). There is ample evidence of the physiological effects of legumes in restricting various metabolic diseases such as diabetes mellitus, coronary artery disease and colon cancer (Tharanathan & Mahadevamma, 2003). Regular

consumption of legumes has been associated with the decreased risk of cardiovascular disease, stroke, Parkinson's, Alzheimer's diseases, liver ailments and cancer (Singh, 2005). Though legumes are a good source of protein, their yield is much lower in comparison to cereals (Razdan & Cocking, 1981) because of the ever-increasing demand for cereals from human population (Siddique, Johansen, Turner, Jeuffroy, Hashem, Sakar, et al., 2012). Legumes are a rich source of B vitamins that comprise riboflavin, thiamin, niacin, pyridoxine and folic acid and they play a vital role in energy metabolism (Rebello, Greenway, & Finley, 2014). They are a rich source of iron and other minerals. However, they also contain antinutritional factors such as proteinase inhibitors, lectin, saponins, phytate etc. that decrease the nutritional value of food by lowering the digestibility or bioavailability of nutrients (Sandberg, 2002). Legumes are rich in lysine, an essential amino acid but are deficient in sulphur-containing aminoacids, methionine and cysteine. Whereas, cereal grains are deficient in lysine but possess adequate amounts of sulphur aminoacids (Singh & Singh, 1992). Due to the lack of knowledge regarding the nutritional composition, large quantities of leguminous seeds remain unexplored (Prakash et al., 2001). New research approaches that depend on biotechnology to improve the utilization of grain legumes will have positive impacts on the nutritional quality of legumes (Duranti & Gius, 1997).

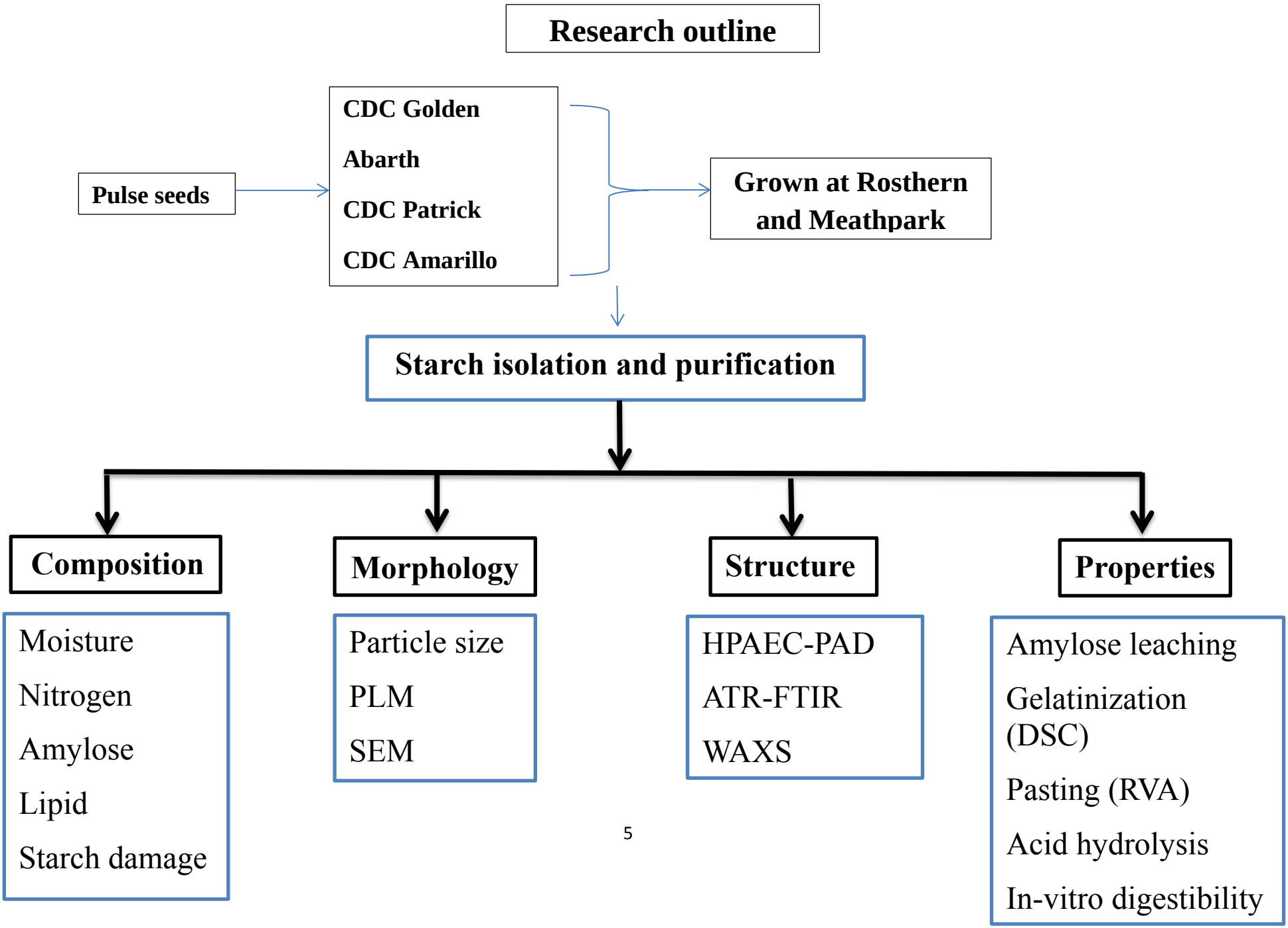
Pulses are the edible seeds of the plants belonging to the legume family. They include dried beans, dried peas, chickpeas and lentils (Curran, 2012). They are grown for many years and are useful in restoring soil fertility, maintaining soil quality (Ganeshamurthy, 2009) and possess several physiological benefits (Rochfort & Panozzo, 2007). They are a rich source of dietary fibre, protein, carbohydrates (Campos-Vega, Loarca-Piña, &

Oomah, 2010), minerals and vitamins necessary for human health (Bushra, Bhanu, Kiran, & Pramod, 2015) and are also low in fat (Longnecker, 2000). They have several bioactive substances that cannot be termed as nutrients, but exert metabolic effects on humans (Champ, 2002). Canadian pulse production has increased from about 1 million tonne in the early 1990s to 5.9 million tonnes in 2015. Canada exported 6 million tonnes of pulses in 2015, valued nearly $4.2 billion (Pulse Canada, 2016). With approximately 15000 pulse growers, Saskatchewan holds an important place in the province's agricultural industry (Saskatchewan pulse growers, 2016). Among pulses produced worldwide, dry beans, peas, chick peas and lentils contribute about 46, 26, 20 and 8%, respectively. Canada is the world largest producer of field peas (3.4 million tonnes (MT)) followed by China (1.6 MT), Russia (1.4 MT), USA (7.4 kilotonnes (kT)) and India (600 kT) (http:// faostat.fao.org/).

Increase in consumption of pulses throughout the world is attributed to their high nutritional value, being low in calories and glycaemic index (Rizkalla, Bellisle, & Slama, 2002). Pulses also help in controlling cholesterol and triglyceride levels (Asif, Rooney, Ali, & Riaz, 2013) and thus intake of pulses has been associated with reduced risk for cardiovascular diseases, diabetes, bone health and weight management (Anderson & Major, 2002). Pulse intake has been associated with lowering serum cholesterol and increasing saturation levels of cholesterol in the bile (Singh & Basu, 2012).

Starch is a versatile raw material having a variety of applications and the increasing demand of starches has resulted in the interest in developing new sources of this polysaccharide in food industry (Betancur, Ancona, Guerrero, Camelo Matos, & Ortiz,

2001). Legume starch accounts for 22-45% of the seed and is the main storage component. The property of legume starches being resistant towards hydrolysis is considered important by nutritionists because they exhibit a lower glycaemic index than cereals (Hoover & Sosulski, 1991).Pulses that comprise peas, lentils, beans and chickpeas constitute 18.5-30% protein and 14.4-26.3% fiber on a dry weight basis (Toews & Wang, 2013). Because of the high cost of isolation, high retrogradation rates and less information available on the amylose and amylopectin structure, pulse starches are not widely used in food industries (Chibbar, Ambigaipalan, & Hoover, 2010). The objectives of this study are to isolate starch from eight newly released cultivars of field peas (*Pisum sativum*) grown at Rosthern and Meathpark in Saskatchewan, Canada and to determine the composition, molecular structure, gelatinization parameters, stability towards heat and shear, kinetics of acid hydrolysis, starch nutritional fractions, and the rate and extent of retrogradation.


Research outline
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Chapter 2

Literature review

2.1 Starch

Starch is the main carbohydrate reserve in plants found in photosynthetic and nonphotosynthetic tissues. Starch that is present in the chloroplasts of leaves is referred to as “transitory starch” because of the diurnal rise and fall of its levels in these tissues. Transitory and reserve starch can be classified based on their physical properties such as size, shape and composition. Transitory starch granules are smaller whereas reserve granules have species-specific shapes. Transitory starch is made entirely of the branched amylopectin but reserve starch has significant amounts of amylose in addition to amylopectin (Slattery, Kavakli, & Okita, 2000).

Starch constitutes two-thirds of the carbohydrate caloric intake of most humans (Whistler & Daniel, 1978). Starch is an interesting polymer in food industry (Ayoub, Ohtani, & Sugiyama, 2006). It is biodegradable, edible and non-reliable on fossil sources (García, Famá, Dufresne, Aranguren, & Goyanes, 2009) and accounts for approximately 70% of the dry weight of cereal seeds (Hannah & James, 2008). Cereal grains, tuber and legume seeds contain starch, but the extent of digestibility depends on the plant type, physicochemical properties of starch and processing/storage conditions (Liu, Donner, Yin, Huang, & Fan, 2006).

Starch is an important functional food biopolymer contributing to the characteristic properties of food products made from cereals, rice, potato and maize and is also added as a functional ingredient to several products such as sauces, puddings, confectionery, etc (Hermansson & Svegmark, 1996).

2.2 Starch biosynthesis:

Biosynthesis of starch involves several steps and is a complex process. Starch is synthesized in leaves from photosynthetically fixed carbon during the day and mobilized at night. Though starch is synthesized transiently in organs such as meristems and root cap cells, its major site of accumulation is in storage organs (Martin & Smith, 1995). Sucrose that is derived from photosynthesis is the initial point of alpha-glucan deposition and it is converted to uridine diphosphate glucose and fructose by sucrose synthase in the cytosol. The UDP-glucose is then converted to glucose-1-phosphate by UDP-glucose pyrophosphorylase in the presence of pyrophosphate which is subsequently converted to glucose-6-phosphate by phosphoglucomutase. The glucose-6-phosphate is then translocated across the amyloplast membrane and subsequently converted to glucose-1-phosphate by phosphoglucomutase. The resulting glucose-1-phosphate may be either translocated directly into the amyloplast or converted to and then translocated as adenosine diphosphate glucose that is generated as a consequence of ADP-glucose pyrophosphorylase activity in the presence of ATP. ADP-glucose provides glucose residues for the biosynthesis of amylose and amylopectin. Starch synthases are classified as “granule bound” and “soluble” and these add glucose units to the non-reducing ends of amylose and amylopectin. Granule bound starch synthase can extend malto-oligosaccharides to form amylose and the soluble starch synthase is responsible for the amylopectin synthesis (Tester, Karkalas, & Qi, 2004).

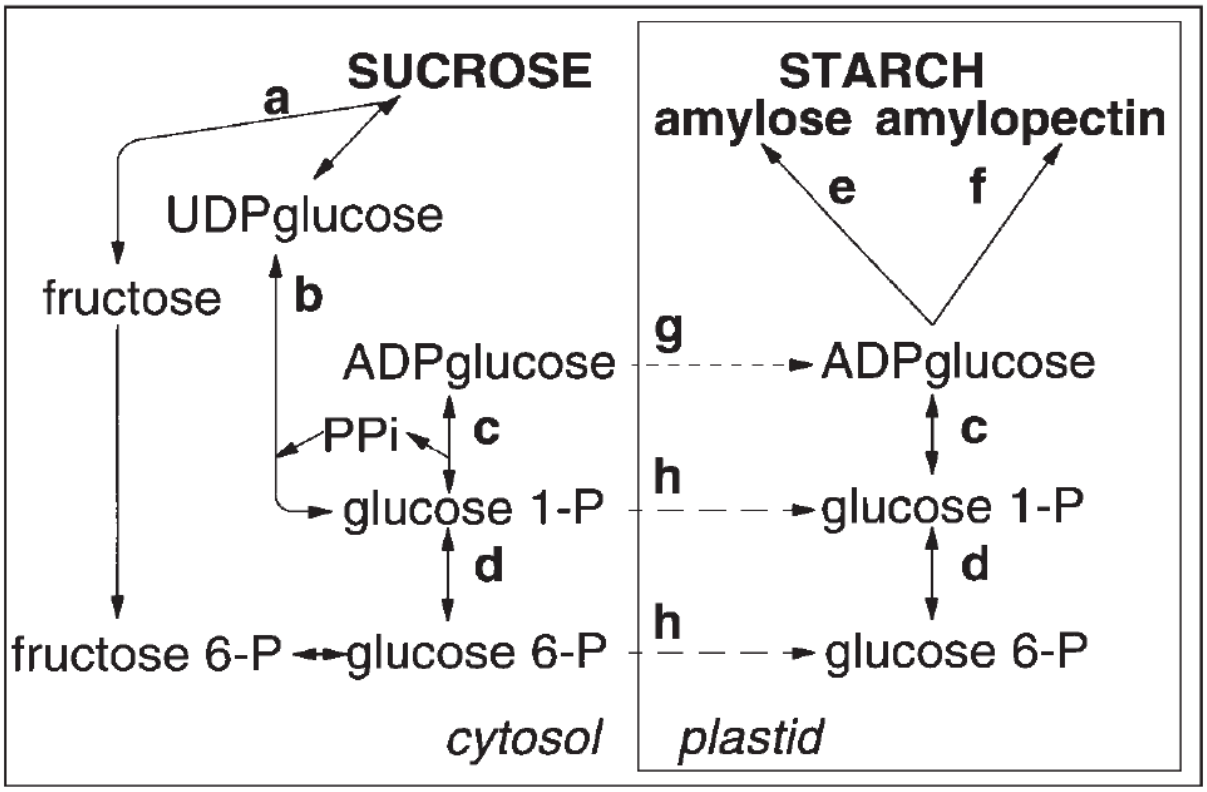

Figure 2.1: The major metabolites and enzymes involved in the conversion of sucrose to starch in storage organs. Enzymes are: a) Sucrose synthase, b) UDPglucose pyrophosphorylase, c) ADPglucose pyrophosphorylase, d) Phosphoglucomutase, e) starch synthase, f) starch synthase and starch branching enzyme, g) ADP glucose transporter, h) hexose phosphate transporter, PPi – inorganic pyrophosphate (Source: Smith, Denyer, & Martin, 1997, reproduced with permission from Annual Reviews).

2.3 Granule morphology and size

The size of the starch granules generally range from 1 to 110 μm (Singh, Singh, Kaur, Sodhi, & Gill, 2003) with varied shapes (spherical, lenticular, polyhedral and irregular) and size distributions (unimodal and bimodal) (Dhital, Shrestha, & Gidley, 2010). Majority of the tuber and root starches have simple granules but cassava and taro starches contain a mixture of simple and compound granules (Hoover, 2001). The width of the starch granules of wheat, rice, barley and potato starch were reported to be 22, 8, 8 and 38 μm, respectively (Palmer, 1972 ; Svihus, Uhlen, & Harstad, 2005). The size of the corn starch granules varied from 3.6 to 14.3 μm, whereas the potato starch granules were flattened ellipsoids with size in the range of 14.3 to 53.6 μm and the size of tapioca starch granules was between 7.1 and 25 μm (Mishra & Rai, 2006). Pulse starches are oval, round, spherical, elliptical or irregular with width in the range of 5 – 55 and 5 – 70 μm in length (Chibbar, Ambigaipalan, & Hoover, 2010).

2.4 Molecular architecture of starch:

The structure of starch in a grain can be categorized into six levels that range in scale from nanometer to millimeter.

Level 1: Individual branches

Individual linear branches is the lowest level wherein the α-D-glucopyranosyl units are linked by α(1→4) glycosidic linkages and the branches comprise two categories: amylopectin, whose average degree of polymerization (DP) is approximately 17-25 and amylose, where it is 10^3-10^4 (Gilbert, Wu, Sullivan, Sumarriva, Ersch, & Hasjim, 2013).

Level 2: Whole starch molecules

This is the structure of the branched molecules. Amylopectin is responsible for the architecture of starch granules and it influences various physicochemical properties. It is a highly branched structure that is composed of A-chains, which do not carry any other chains, B-chains that carry other chains through 1→6 branches and C-chain that has the reducing end (Laohaphatanaleart, Piyachomkwan, Sriroth, & Bertoft, 2010).

Level 3: Lamellar structure

The crystallinity of the granule is attributed to the double helices formed by amylopectin branches and amylose is present in the amorphous layers of growth rings. The crystalline lamella is composed of amylopectin double helices that are packed in a parallel fashion. The amylopectin branch points are present in the amorphous zones (Jacobs & Delcour, 1998). The blocklet level of organization describes the organization of amylopectin

lamellae into spherical blocklets that has diameters ranging from 20 to 500 nm (Baker, Miles, & Helbert, 2001). The blocklets are composed of partially crystalline amylopectin with branches of amylopectin molecules that form the crystalline part of the granule and are found embedded within the amorphous amylose matrix (Ridout, Parker, Hedley, Bogracheva, & Morris, 2004). AFM (Atomic force microscopy) study bears out the observations of the 'blocklet' structure of starch (Gallant, Bouchet, & Baldwin, 1997). The blocklet structure is similar in shape but varies with plant size and is continuous throughout the granule. The size of the blocklets may not correspond to their granular sizes and the thickness of growth (Tang, Mitsunaga, & Kawamura, 2006). An important benefit of using AFM is that minimum starch preparation is sufficient to obtain information on the internal structure of starch granules (Parker, Kirby, & Morris, 2008).

Level 4: Granules

The hierarchical structure of granules can be observed by light and electron microscopy. Several concentric layers of growth rings extend from the hilum towards the surface. The growth rings, which are 120-400 nm in thickness, contain alternating crystalline and amorphous regions of higher and lower density. The higher density regions possess a lamellar structure of alternating crystalline and amorphous layers whereas the amorphous layers has the amylopectin branching points and a disordered conformation of amylose and amylopectin molecules (Copeland, Blazek, Salman, & Tang, 2009).

Level 5: Endosperm

The endosperm is the largest organ in the seed and is covered by a single layer of cells called the aleurone layer (Emes, Bowsher, Hedley, Burrell, Scrase-Field, & Tetlow, 2003). In the seed endosperm, starch is stored as an energy reserve (James, Denyer, & Myers, 2003).

Level 6: Whole grain

This is the final level and is approximately 1 mm in size. It comprises the highest-level structures and the function of granular structure is that it serves as an energy-storage medium for the germinating plant. It also causes the slow release of glucose upon external stimuli. Though amylopectin is sufficient for the starch granule formation, amylose also plays a significant role in the primary stages of granule crystallization (Dona, Pages, Gilbert, & Kuchel, 2010).

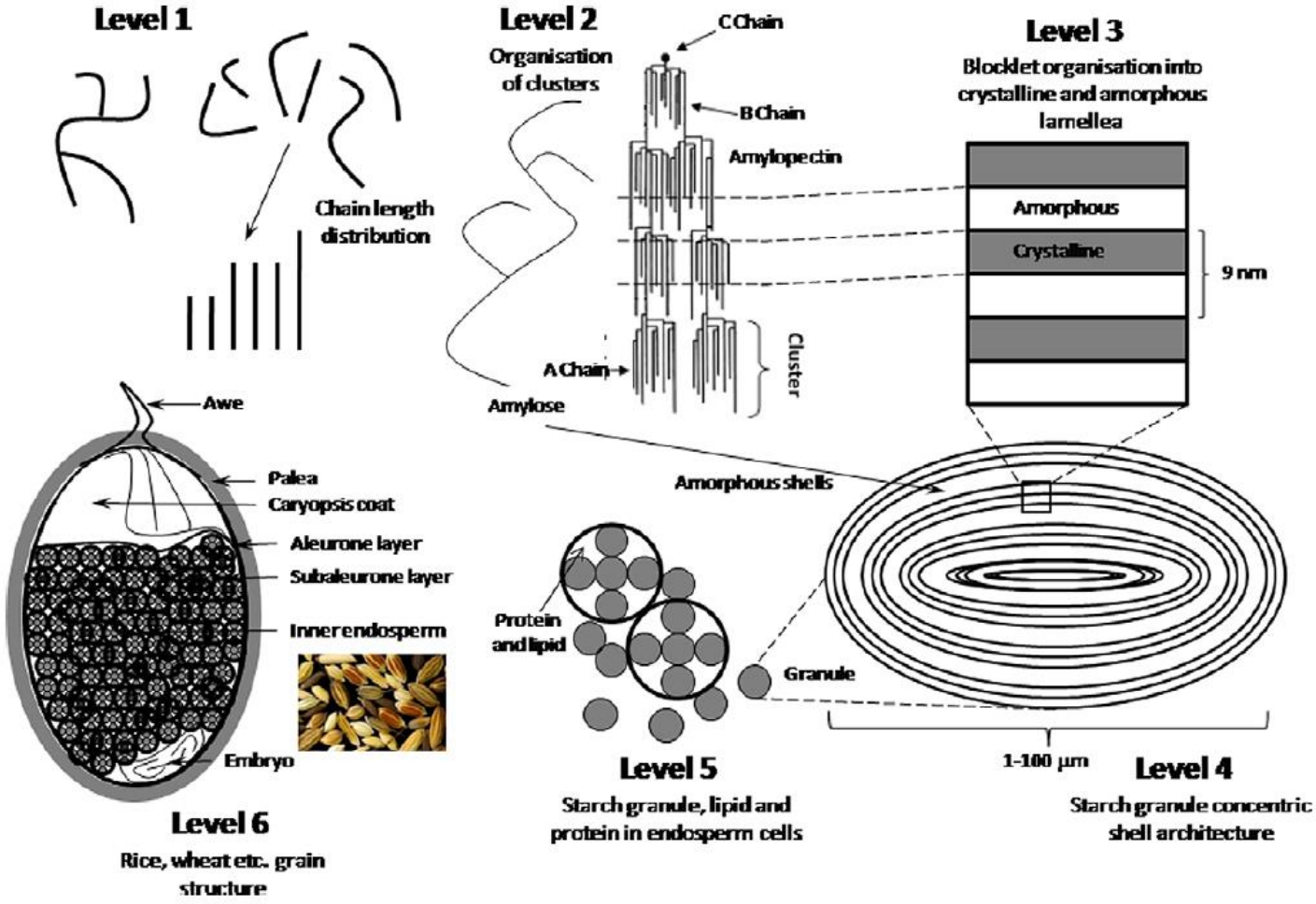

Figure 2.2: Six supramolecular levels of the rice grain, highlighting the microscopic structural contribution of starch (Dona, Pages, Gilbert, & Kuchel., 2010, Copyright Elsevier, reproduced with permission).

2.5 Structure of amylose

Starch is composed of amylose and amylopectin. Amylose is the minor component of the two consisting of α-(1→4) linked D-glucopyranosyl residues although a slight degree of branching in various starch sources has been reported (Hizukuri, Takeda, Yasuda, & Suzuki, 1981). The amylose content in pulse starches range from 24-88%. Because of the differences in growth location, physiological state of seed, cultivar differences and various methodologies used for the determination, it becomes difficult to compare the amylose content among and between the pulse starches (Chibbar, Ambigaipalan, & Hoover, 2010). The molecular weight of amylose ranges approximately from $1 \times 10^5 - 1 \times 10^6$ and has a degree of polymerization of 324-4920 containing 9-20 branch points (Tester, Karkalas, & Qi, 2004). Amylose is formed of anhydroglucose units in the $^4C_1$ chair conformation and six monosaccharide units are found in one turn of a left-handed helix. The hydroxyl groups are located towards the exterior of the helices that allows interaction with polar solutes and the interior of the amylose helices is hydrophobic (Bergthaller, Hollmann, & Johannis, 2007). Amylose exists as a flexible random coil containing left-handed helical segments that are more pronounced at low hydration levels (López, de Vries, & Marrink, 2012). Amylose molecules have a tendency to approach and bond together due to the presence of hydroxyl groups and its linear structure (Kang, Zuo, Hilliou, Ashokkumar, & Hemar, 2016). On the basis of X-ray diffraction studies, it was proposed that the amylose is organized as left-handed helices having outer diameters of 13Å and a pitch of 8Å (Yu, Houtman, & Atalla, 1996). Amylose content can be quantitatively determined by the formation of a helical complex between amylose and

iodine that results in the formation of a typical blue colour. Polyiodide ions such as I_3^- and I_5^- interact with amylose forming single left handed V- type helices. Also the hydrocarbon portion of monoglycerides and fatty acids interact with amylose to form a V-helix complex (Hoover, 2001). Light-absorption spectroscopy can be used to monitor the blue colour of amylose-iodine complex. Hence, this tool is used in combination with circular dichroism to study the complex formation (Saenger, 1984). The conformation of amylose in solution has been investigated for a long time. Three molecular models : a random coil with no helical structure, an interrupted helix with alternate coil portions and helical sequences and a continuously bending helix have been proposed (Norisuye, 1996). For double helix formation in a pure oligosaccharide solution, a minimum chain length of DP 10 is required (Pérez & Bertoft, 2010).

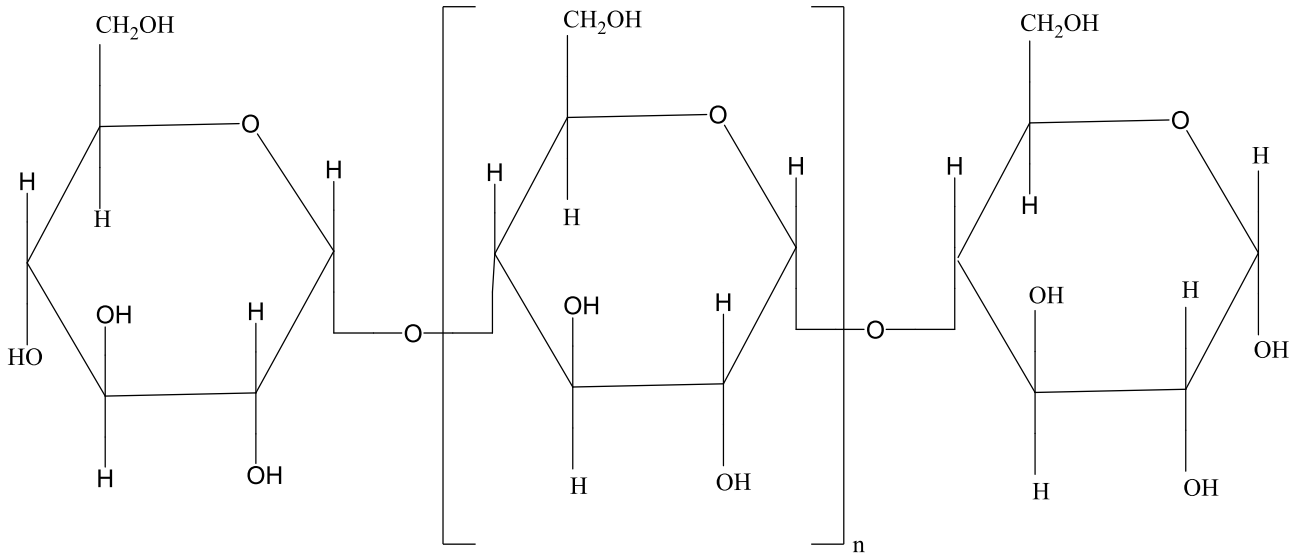

Figure 2.3: Schematic diagram of amylose

2.5.1 Location of amylose:

The location of amylose in native starch granule is still under discussion but it is thought to be present primarily in the amorphous, less-crystalline regions (Jobling, 2004). Amylose was believed to be present in the amorphous portion of the granule which was supported by the results indicating the presence of blue-staining rings when starch granules of low-amylose potato tubers were stained with iodine (Denyer, Johnson, Zeeman, & Smith, 2001). Based on the small-angle X-ray scattering techniques, it was reported that amylose is primarily concentrated in the amorphous growth rings and the reason for decrease in crystallinity is attributed to the interaction of amylose and amylopectin in the amorphous regions (Saibene & Seetharaman, 2010). Considering starch granule properties such as amylose leaching, amylose-iodine complexation, DMSO solubilisation and V-complex formation, amylose is proposed of being separated from amylopectin in the case of normal maize starch and being interspersed with amylopectin in potato starch (Jane, 2006).

Studies on maize, pea and barley starch granules indicated that amylose molecules disrupt the structural order of amylopectin clusters (Atkin, Cheng, Abeysekera, & Robards, 1999). Jane and Shen (1993) proposed that amylose is more concentrated in the periphery of the potato starch granule. However, Tatge, Marshall, Martin, Edwards, and Smith (1999) reported the presence of amylose in the central region of the potato starch granule. The presence of amylose in the amorphous and/or crystalline regions varies with the botanical origin of starch. For instance, amylose is concentrated in the amorphous region in wheat starch whereas in potato starch, it is found partly co-crystallized with

amylopectin (Oates, 1997). Cross-linking reactions were performed on intact native starch granules to investigate whether amylose molecules are interspersed with amylopectin or are found in the form of bundles (Jane, Ao, Duvick, Wiklund, Yoo, Wong et al., 2003). Experiments carried out with cross-linking agents on potato and corn starch indicated that individual amylose molecules are interspersed among the amylopectin molecules and not grouped together (Wang, Blazek, Gilbert, & Copeland, 2012).

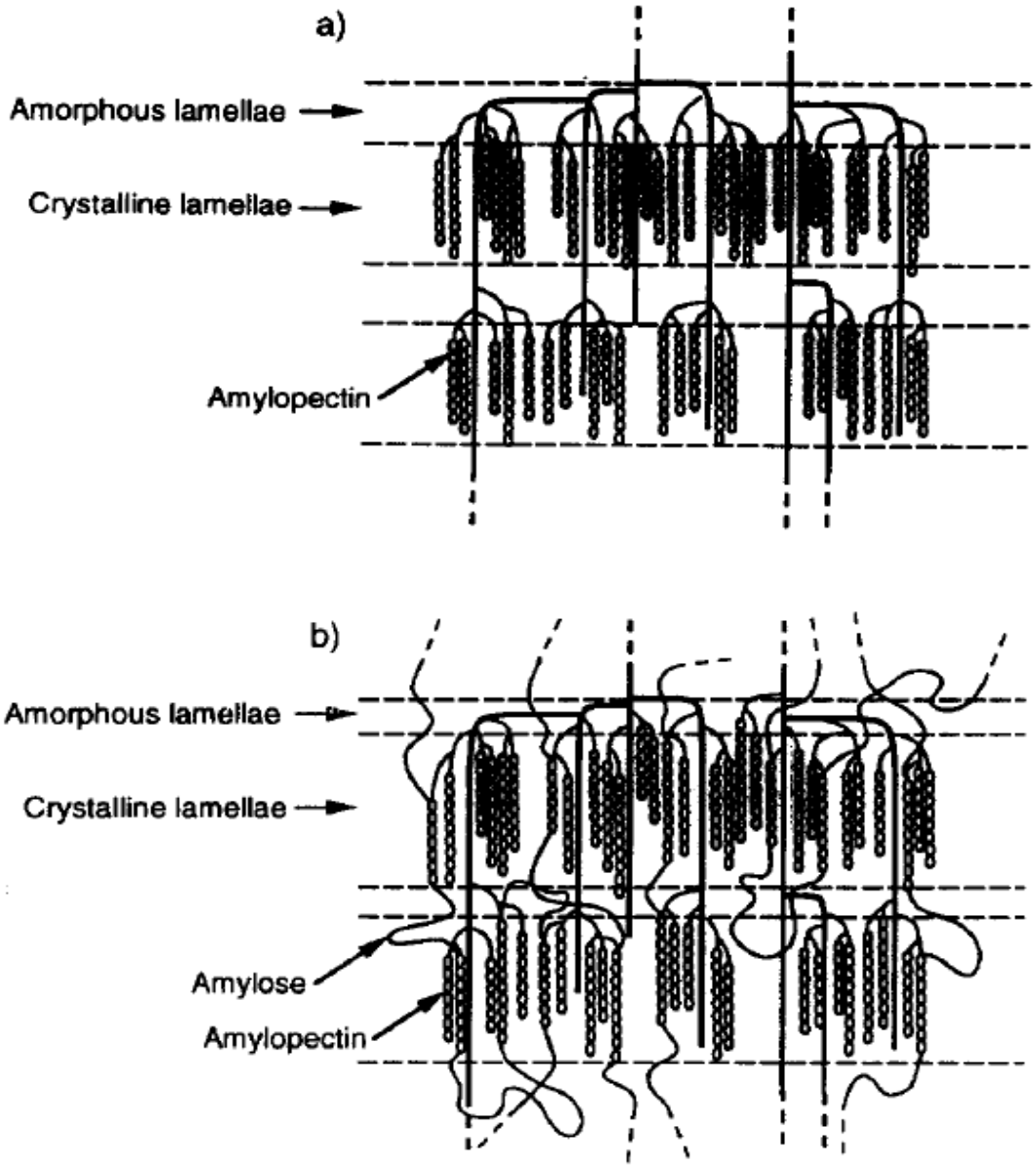

Figure 2.4: Mechanism outlining the role of amylose in disrupting the packing of amylopectin double helices within the crystalline lamellae: (a) Amylopectin structure with no amylose present (b) The co-crystallization of amylose with amylopectin pulls a number of amylopectin chains out of register (Jenkins & Donald, 1995, Copyright Elsevier, reproduced with permission).

2.5.2 Amylose inclusion complexes:

The ability of the linear amylose fraction to form inclusion complexes with a number of ligands is one of the characteristic features of starch. The ligands pass into the helical cavities of the amylose molecules forming molecular inclusion complexes. Amylose undergoes a coil to helix transformation in the presence of ligand molecules that enhances the helical aggregation to partially crystalline V structures (Szezodrak & Pomeranz, 1992). This complex decreases the water solubility and susceptibility of starches to enzyme digestion (Kaur & Singh, 2000) and also modifies the rheology of starch (Singh, Singh, & Saxena, 2002). The longer the lipid chain, longer the amylose chain has to be for lipid complexation. Longer lipid chain lengths tend to be more hydrophobic and hence less soluble in water (Putseys, Derde, Lamberts, Goesaert, & Delcour, 2009). Three polymorphs of amylose, A, B and V forms exist and the V-form requires a complexing ligand. No hydrogen bonding exists between consecutive turns of the helices in A and B forms whereas in the V-form, amylose forms a helix with a large cavity in which various ligands are present (Pethrick & Song, 2013). The V-forms have a pitch of about 8Å per turn, whereas the A and B forms have a pitch of about 21Å and is characterized by the absence of internal cavity (Snape, Morrison, Maroto-Valer, Karkalas, & Pethrick, 1998). Amylose complexes with polar and nonpolar compounds (Jovanovich & Añón, 1999). There are some forces holding the helix conformation. Intramolecular bonds occur between the helix turns and intermolecular forces stabilize the interactions between amylose and its ligand. The formation of hydrophobic interactions is favoured as the

amylose helix is hydrophilic on the outside and hydrophobic inside (Putseys, Lamberts, & Delcour, 2010).

The formation of amylose-lipid complex depends on several factors such as degree of polymerization, pH of the solution, complexation temperature and the complexed lipid structure (Zabar, Lesmes, Katz, Shimoni, & Bianco-Peled, 2009). For starches with normal amylose content, phase transition that occurs during gelatinization is observed as a single endotherm at around 55-75°C whereas amylose-rich endotherms exhibit a broad endotherm between 80 and 130°C with a second reversible endothermic transition noticed at 100°C for lipid-containing cereal starches that is attributed to the melting of amylose-lipid complex (Le Bail, Bizot, Ollivon, Keller, Bourgaux, & Buléon, 1999).

Various methods have been used to study the starch-lipid complexes. For instance, X-ray diffraction is used to measure crystallinity and DSC to observe the melting-transition characteristics and stability of the complexes (De Pilli, Derossi, Talja, Jouppila, & Severini, 2011). The lipid-amylose complexation is a modification occurring during extrusion cooking as it influences the paste viscosity and extrudate texture (De Pilli, Legrand, Derossi, & Severini, 2015). DSC is a useful method in gaining insight into the properties of starch. Starch-lipid interaction influences gelatinization and restricts recrystallization of gels (Cieśla & Eliasson, 2007). For example, in wheat flour, starch is one of the main constituents and they may interact with water, lipids, sugars and hydrophilic macromolecules which have an effect on its properties (Jovanovich & Añón, 1999).

Starch and lipids play a vital role in functional interactions in food systems (Tang & Copeland, 2007). Starch-lipid complexation impacts the formation of resistant starch, starch pasting and gel texture behaviour. For example, lipids complexing with amylose on the granule surface restricts swelling (Zhou, Robards, Helliwell, & Blanchard, 2007). Polar lipids play an important role in influencing the starch behaviour towards the development of viscous and gelling properties (Godet, Bouchet, Colonna, Gallant, & Buleon, 1996). The amylose-lipid complexes have several important applications such as emulsifiers in delaying bread staling, in nanotechnology for helical wrapping of carbon nanotubes and in biotechnology for artificial chaperoning of proteins (Gelders, Vanderstukken, Goesaert, & Delcour, 2004).

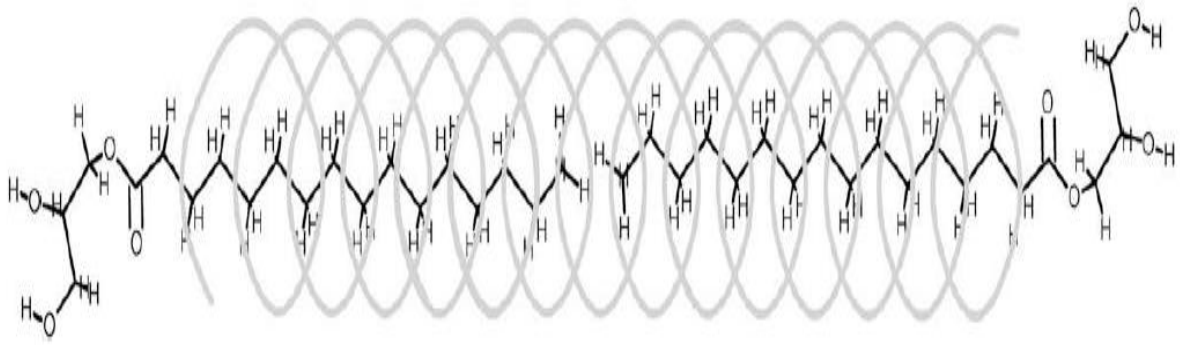

Figure 2.5: Schematic representation of amylose complex with two monopalmitin molecules (Copeland et al., 2009, Copyright Elsevier, reproduced with permission).

2.5.3 Determination of amylose content:

Amylose content determination is important in starch characterization because it influences the functional properties of starch in food industry and other applications (Knutson, 2000). Near-infrared spectroscopy is a simple method for determining the amylose content in starch because it is rapid and non-destructive. In addition to that, it requires minimal or no sample preparation (Fertig, Podczeck, Jee, & Smith, 2004). Size exclusion chromatography has also been used in which the molecules are separated according to their hydrodynamic radius (Gérard, Barron, Colonna, & Planchot, 2001). The amylose content of starches can also be determined by a calorimetric procedure that involves the formation and melting of the lysolecithin complex (Kugimiya & Donovan, 1981).

The blue complex of amylose and iodine has been the subject of investigation since its discovery (Yamagishi, Imamura, & Fujimoto, 1972) and the physicochemical properties are studied (Takahashi & Ono, 1972). Triiodide ion required for the initiation of amylose-iodine complex formation forms spontaneously when iodine is dissolved in DMSO. Starch, when dissolved in DMSO containing iodine and diluting with water forms the amylose-iodine complex whose absorbance is measured at 600 nm. This forms the basis for the estimation of amylose content (Knutson & Grove, 1994). Amylose forms highly coloured iodine complexes because of the long helices it can form whereas the ability of the amylopectin to form complexes and bind iodine is weaker because of its shorter chain length (Suortti, Gorenstein, & Roger, 1998).

Though a number of techniques are used to determine the amylose content of starch such as iodine calorimetry, potentiometric titration (Duan, Donner, Liu, Smith, & Ravenelle, 2012), amperometry (Gibson, Solah, & McCleary, 1997), size exclusion chromatography and concanavalin A precipitation, the results can vary noticeably because each technique measures a different property that is converted to a purported amylose content (Vilaplana, Hasjim, & Gilbert, 2012) and there are certain drawbacks associated with each method. NIR procedure requires standardisation for each material, HPSEC uses expensive columns and DSC is applicable only for the analysis of crystal structure in starch but it can be affected by heat treatment. Though iodine calorimetry is widely used in the amylose content determination, the accuracy is limited because of interference of amylose with lipids (Wang, Yu, Xu, Yang, Jin, & Kim, 2011).The enzymatic method is very specific, but may lead to underestimation in materials that contain starch resistant to gelatinisation or enzyme hydrolysis (Stawski, 2008).

2.6 Structure of amylopectin:

Amylopectin is an extensively branched component in comparison with amylose. It is composed of α-D-glucopyranosyl residues linked by (1→4) linkages and 5-6% of (1→6) bonds at the branch points (Hizukuri, 1985) and is the major component of starch contributing to the architecture of the starch granules (Bertoft, 2007b). It has a molecular weight of about 10^8 and a degree of polymerization (DP) that is more than one million. In pulses, the average chain length and the proportion of chains with DP 6-12, 13-24, 25-36 and 37-50 range from 17-27, 16-27, 28-60, 14-56 and 4.5-19.4, respectively (Chibbar, Ambigaipalan, & Hoover, 2010). The organization of the unit chains in amylopectin is important in gaining insight into the structure and architecture of the macromolecule. Currently, two major structural models exist: cluster model and the building block backbone model. The primary difference between the backbone and traditional models is the different visualization of the organization of chains within amylopectin (Chauhan & Seetharaman, 2013). The cluster model proposes that the short unit chains with less than approximately 36 glycosyl units are organized into clusters and the long chains interconnect them. However, the building block backbone model proposes that the clusters are built up from still smaller structural units referred to as building blocks (Vamadevan & Bertoft, 2015) and the long chains (>35 glucosyl units) form the backbone and branched building blocks that are smaller than clusters and are outspread along the backbone forming the structural unit (Peymanpour, Marcone, Ragaee, Tetlow, Lane, Seetharaman et al., 2016).

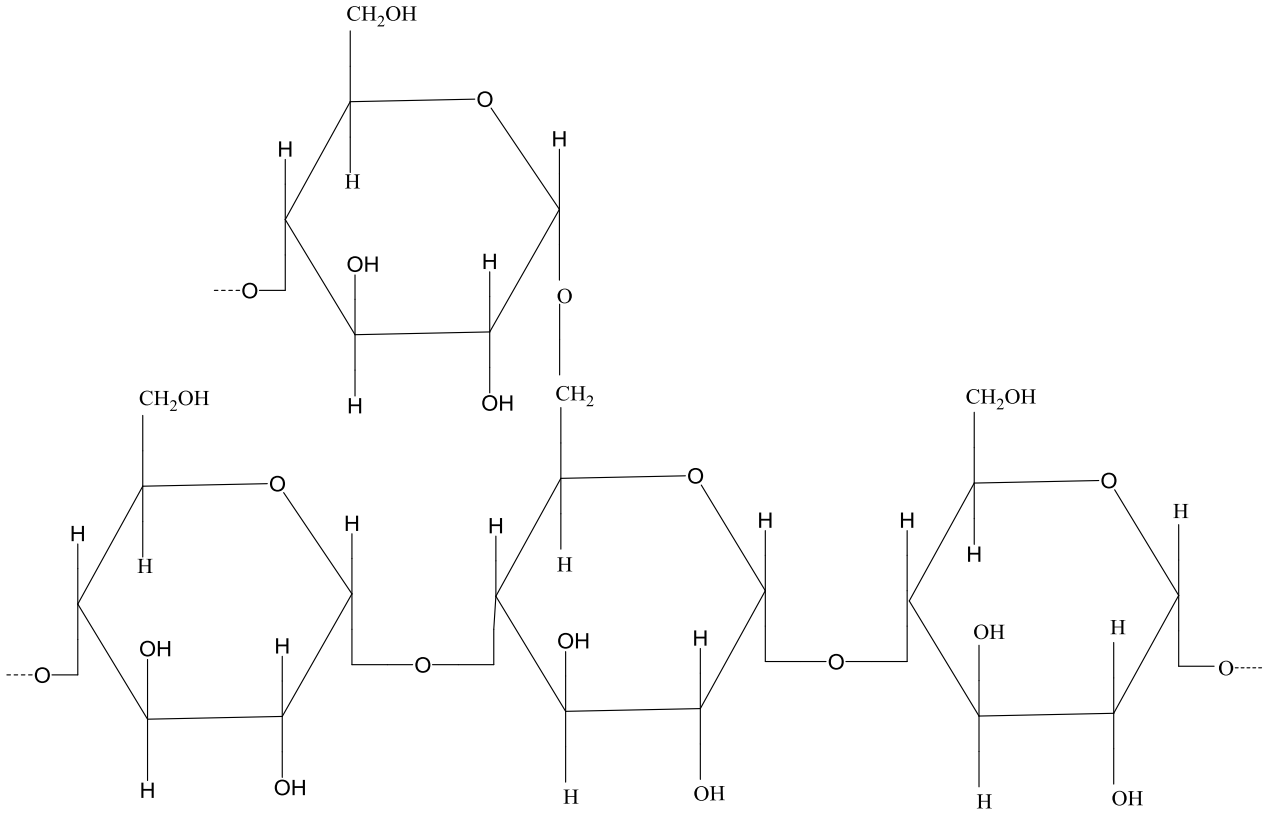

Figure 2.6: Schematic diagram of amylopectin

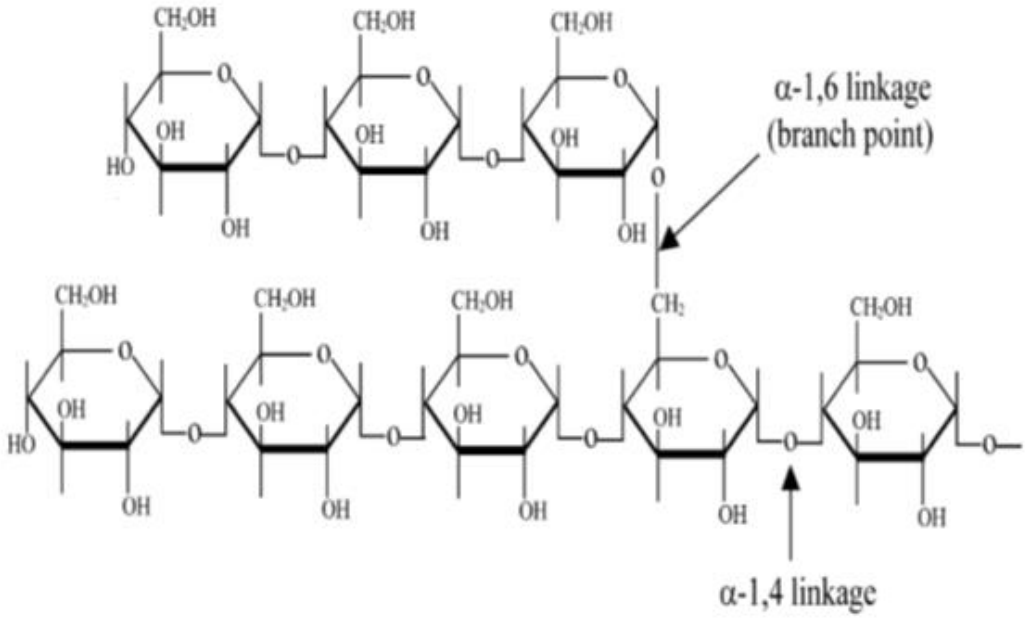

Figure 2.7: The α-(1,4) and α-(1,6) linkages between the glucosyl units present in the amylopectin and amylose of starch (Tharanathan, R., & Mahadevamma, S., 2003, Copyright Elsevier, reproduced with permission).

2.6.1 Cluster model of amylopectin

One of the most widely accepted models of amylopectin was that proposed by Hizukuri in 1985. The cluster model can be described in the following ways: 1. Formation of crystalline double helices as physical clusters from exterior linear region of amylopectin, 2. Distribution of 1,6 branch points with periodic variation in branch point density (Thompson, 2000). Based on the pattern of substitution and chain lengths, the amylopectin branches may be classified into A, B and C chains (Hizukuri, 1985). A-chains are unsubstituted. They are linked to B-chains and do not carry any other chains. B-chains are substituted by other chains and are further classified as B1-B4 based on the number of clusters they span and the C-chain carries the reducing end group of the molecule (Copeland, Blazek, Salman, & Tang, 2009). The external segments of the clusters within the starch granules form double helices which crystallize into A or B polymorphs. B-type starches have longer average chain lengths and a higher proportion of long chains in comparison to A-type starches. The crystals form 5-6 nm thick lamellae that alternate with amorphous lamellae of 3-4 nm thickness (Bertoft, 2007b). Short chains within the starch granules form clusters and the external segments of chains form double helices that account for the crystalline structure. Short and clustered chains were defined A and B1 chains and the clusters are interconnected through long chains: B2 chains participate in the interlinkage of two clusters and B3 chains form three clusters etc. (Laohaphatanaleart, Piyachomkwan, Sriroth, & Bertoft, 2010).

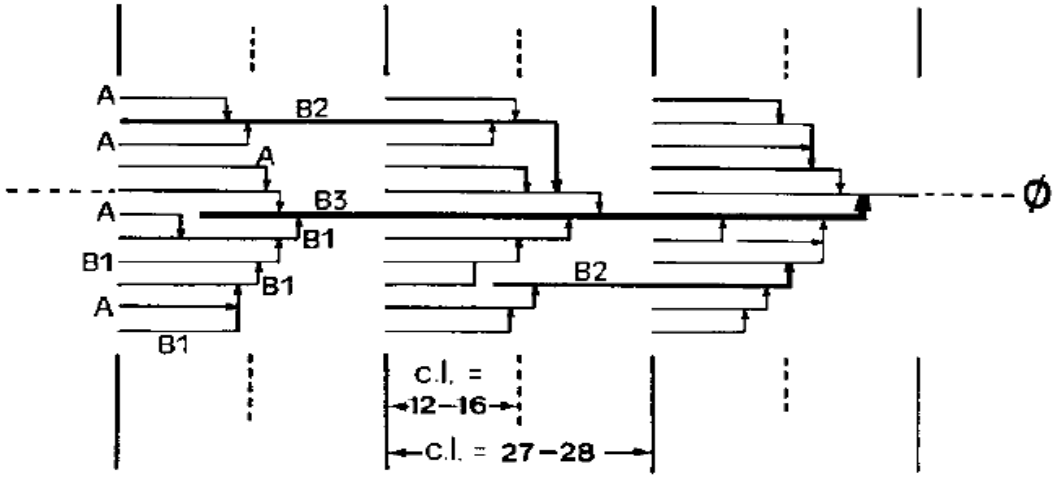

Figure 2.8: Cluster model of amylopectin indicating A, B1-B3 chains, ϕ is the reducing chain-end (Hizukuri, 1986, Copyright Elsevier, reproduced with permission)

2.6.2 Internal unit chains of amylopectin

The amylopectin chains are classified as external and internal. External chains build up the crystalline lamellae whereas the internal chains are present among the clusters of branches in the amorphous lamellae. The entire A-chains are external but the B-chains consist of an external and an internal segment (Bertoft, Piyachomkwan, Chatakanonda, & Sriroth, 2008). The exoacting enzymes, phosphorylase and β-amylase were used to remove the external chains of the cluster. The resulting limit dextrins (named ϕ,β-limit dextrin) had only the internal structure (Bertoft, 2007a). The internal chains are divided into short and long chains. Short B-chains contained two subgroups: the major group at DP 8-25 and a minor 'fingerprint', B_{fp}- group at DP 3-7 (Bertoft, Koch, & Åman, 2012). On the basis of the internal unit chain profiles, amylopectin is classified into four types: Type 1 amylopectin contains only A-allomorph starches with little B2 chains. Type 2 amylopectin consists of more BL-chains. Type 3 amylopectin contains more of the long B3 chains but less B_{fp} chains. Type 4 amylopectin contains a large number of B3 chains and low content of BS chains (Bertoft, Koch, & Åman, 2012).

2.6.3 Blocklet model of amylopectin

Before 1960, it was hypothesized that starch granules consist of crystalline units embedded in amorphous material (Gallant, Bouchet, & Baldwin, 1997). Later, an additional level of structural organization named blocklets was put forward by Gallant and coworkers. The blocklets are envisioned as parcels of crystals distributed within the growth rings (Ridout, Parker, Hedley, Bogracheva, & Morris, 2003). The blocklet concept describes the organization of amylopectin lamellae into spherical blocklets with diameters ranging from 20 to 500 nm, varying with the botanical source of starch (Baker, Miles, & Helbert, 2001). Based on electron microscopy studies, it has been proposed that starch granules contain structures referred as 'blocklets' and they are proposed to contain packets of partially crystalline amylopectin. Atomic force microscopy has been used in the observation of blocklet model as it provides the possibility of imaging under more natural conditions i.e. it does not require treatment with acid or enzyme to cause contrast in the images (Morris, 2004). The blocklet structure is similar in shape but varies with the plant size. The blocklet is continuous throughout the granule. Some defects may occur in the amorphous rings during the blocklet production. An interconnecting matrix is present surrounding the group of blocklets and the growth rings and amorphous rings are not always continuous structures (Tang, Mitsunaga, & Kawamura, 2006).

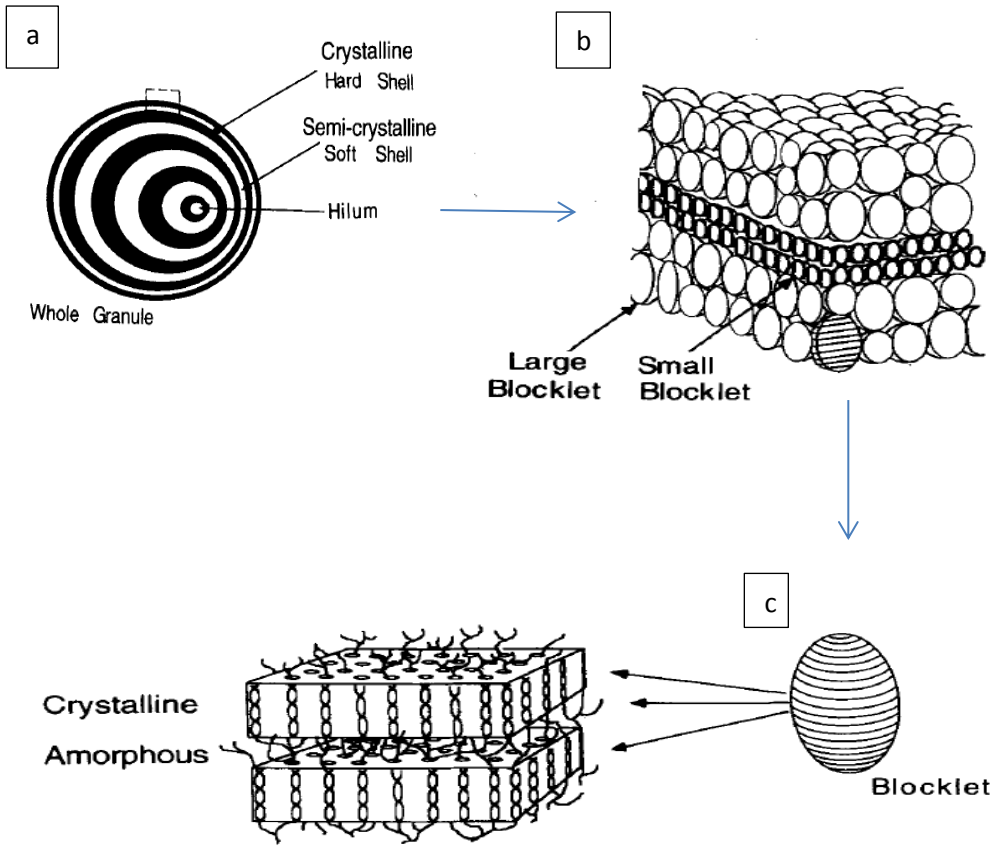

Figure 2.9: Overview of the starch granule structure: a) The lowest level of starch granule organization indicates the alternate crystalline and semi-crystalline shells, b) The blocklet structure is shown, c) One blocklet is shown to contain several amorphous crystalline lamellae (Adapted from Gallant et al., 1997).

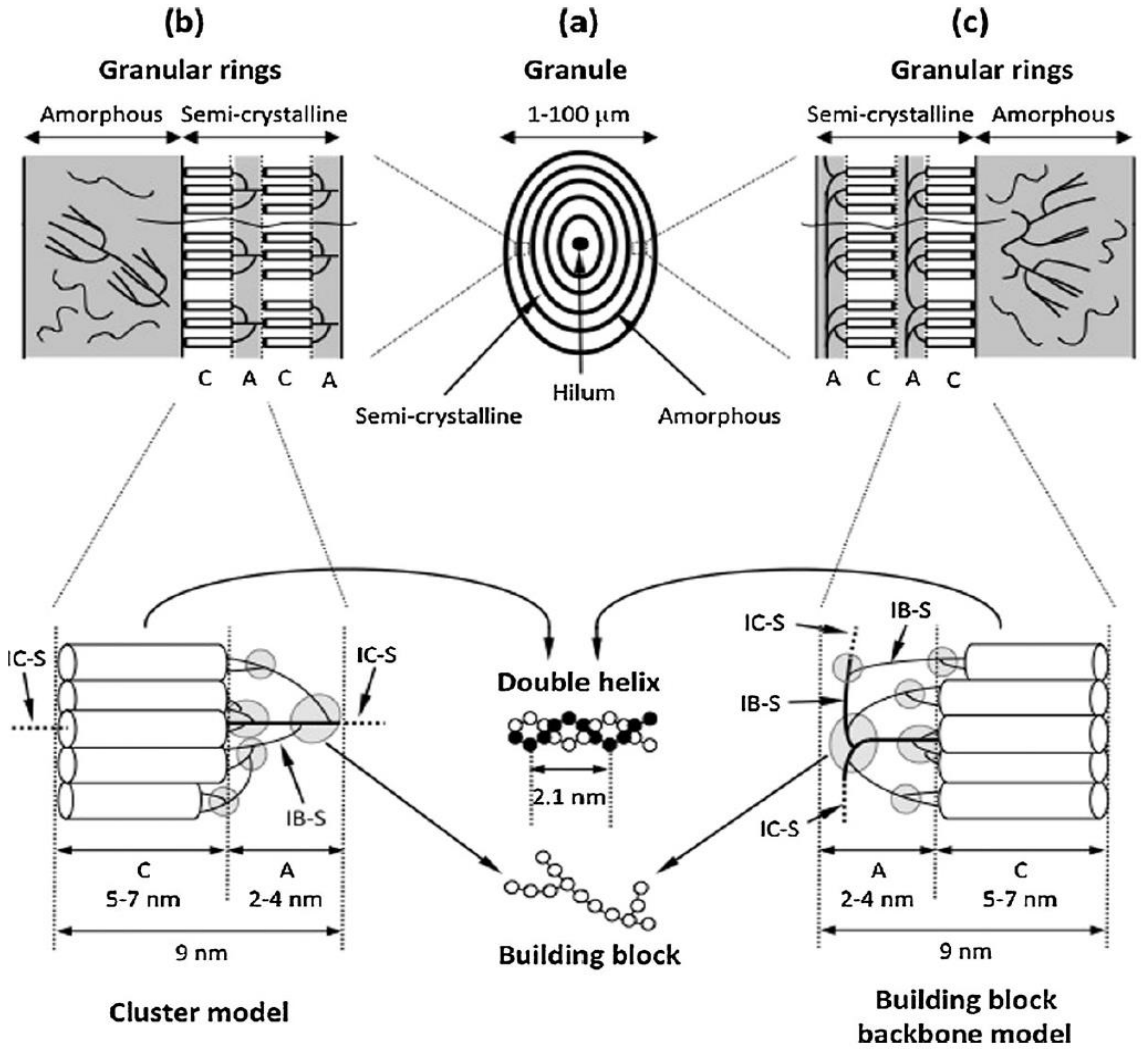

Figure 2.10 : From starch granules to building blocks, a schematic showing different structural levels of starch granules (a) The granule containing alternate regions with a hilum region in the middle, (b) & (c) The arrangement of the semicrystalline rings according to the cluster and building block backbone structure of amylopectin (Vamadevan & Bertoft, 2015, reproduced with permission from John Wiley and Sons).

2.6.4 Analysis of APCLD

Chain-length distribution is one of the key parameters in describing the molecular structure of amylopectin (Bello-Perez, Paredes-Lopez, Roger, & Colonna, 1996) and size exclusion chromatography was used to estimate the chain length which exhibited bimodal distribution: F1 (long B chain) and F2 (short B and A chains) and a correlation was found between the ratio of F2/F1 and weight-average chain length. But SEC could not achieve the separation of individual chains and so high-performance liquid chromatography on an NH_2-bonded silica column with a refractive index detector was used to separate chains up to DP ~ 26. As the major portion of chains is distributed up to DP ~ 100, this technique was replaced by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Hanashiro, Abe, & Hizukuri, 1996). HPAEC-PAD provides information on the amount of the individual unit glucan chains and separation of individual maltosaccharides with DP up to 80 with high resolution could be achieved (Koch, Andersson, & Åman, 1998). The chain-length distributions are divided as follows: A-chains with DP 6-12, B1 chains having DP 13-24, B2 chains with DP 25-36 and B3 chains with DP>37. A-type starches possess amylopectin of more A-chains and B-type starches have fewer A-chains (Jane, Wong, & McPherson, 1997). C-type starches possess amylopectins with both long and short branch chain lengths (McPherson & Jane, 1999). HPAEC-PAD has been used for the analysis of amylopectin chain length distribution (Nagamine & Komae, 1996). The amylopectin chain length distribution (APCLD) is an important factor influencing starch gelatinization properties (Noda, Takahata, Sato, Suda, Morishita, Ishiguro et al., 1998).

2.6.5 Starch crystallinity

Native granular starch is semi-crystalline and can possess different crystalline structures with packed double helices (Rindlava, Hulleman, & Gatenholma, 1997). Investigation of starch crystallinity requires the presence of water as dry starch exhibit a completely amorphous X-ray pattern whereas the crystallinity of B-type starches varies on the basis of water contents (Myllärinen, Buleon, Lahtinen, & Forssell, 2002).

Wide angle X-ray scattering (WAXS) is used in determining the crystal structure and regular molecular arrangements in native and processed starch (Frost, Kaminski, Kirwan, Lascaris, & Shanks, 2009). Based on the botanical origin and composition, starch granules exhibit three types of X-ray diffraction patterns. Cereal starches exhibit an 'A' type diffraction pattern whereas the tuber, root, high-amylose and retrograded starches exhibit a typical 'B' type X-ay pattern with broad and weak peaks and two main reflections at 5.5 and 17° 2θ angles. The 'C' type diffraction pattern which is the characteristic of most legume starches is believed to be a superposition of 'A' and 'B' patterns respectively (Hoover, 2001). The 'A' and 'B' types of starch crystals exhibit differences in the geometry of the unit cell with variations in the bound water attached to the double helices (8 and 36 water molecules, respectively) (Genkina, Wikman, Bertoft, & Yuryev, 2007). Another polymorph found is the V-type which arises from single amylose helices that are complexed with lipids (Lopez-Rubio, Flanagan, Gilbert, & Gidley, 2008).

The X-ray diffraction patterns are useful in differentiating various native starches and in predicting the changes in crystallinity brought about physical or chemical treatments (Singh, Ali, Somashekar, & Mukherjee, 2006). The climatic conditions during plant growth and genetic control are also important factors influencing the crystalline nature of starch (Buléon, Colonna, Planchot, & Ball, 1998). Factors influencing the differences in relative crystallinity among starches are crystallite size, orientation of double helices within the crystallites, average chain length of amylopectin and the mole percentage of short chain fraction of amylopectin (Gunaratne & Hoover, 2002). In pulse starches, the proportion of B-unit cells range from 26 to 92.2% and the crystallinity ranges from 17 to 34%. Because of the differences in moisture content of the starches and the methodology used in calculating crystallinity, it is difficult to compare the crystallinity of various pulse starches (Chibbar, Ambigaipalan, & Hoover, 2010).

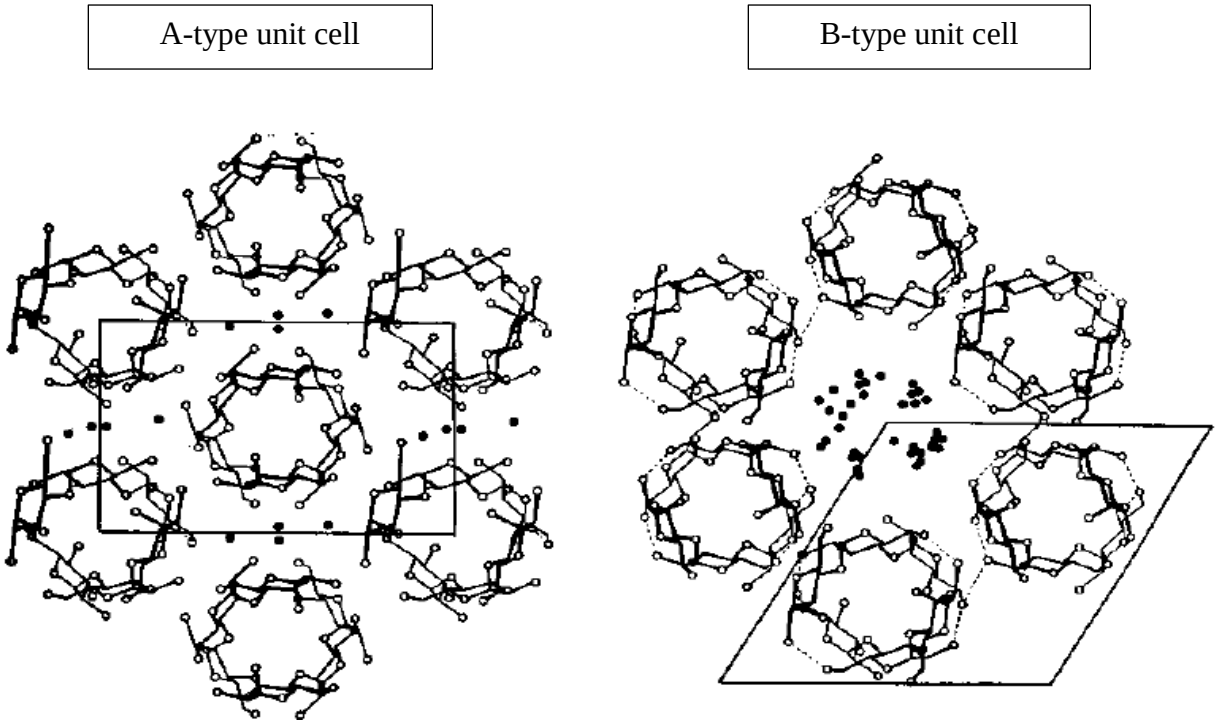

Figure 2.11: Double helices arrangement of A-type and B-type crystallites in starch (Wu and Sarko, 1978, Copyright Elsevier, reproduced with permission)

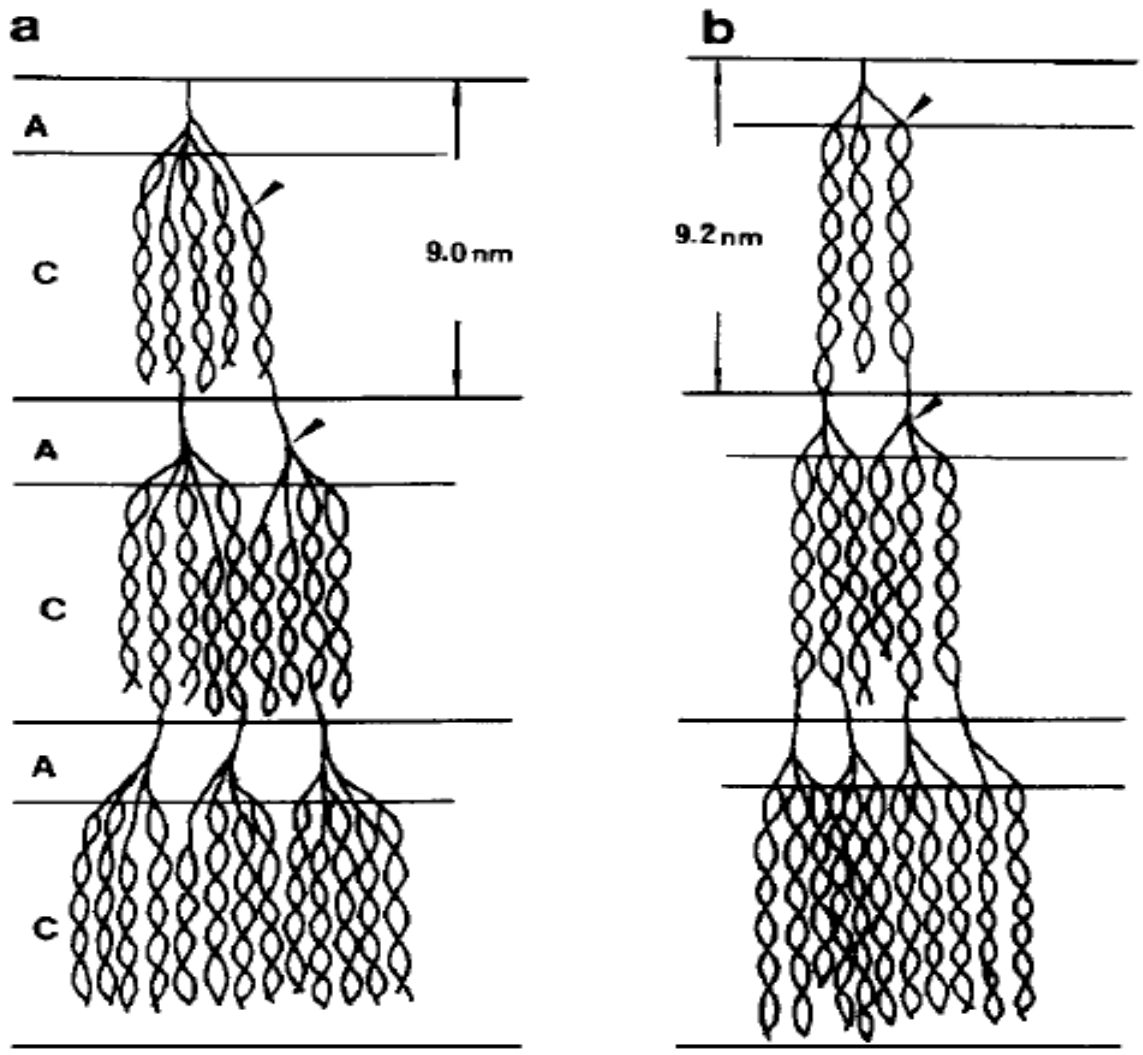

Figure 2.12: Proposed models for the branching patterns of a) waxy maize starch which displays the A-type X-ray pattern and b) potato starch, which displays the B-type X-ray pattern. 'A' and 'C' refers to the amorphous and crystalline regions (Jane, Wong and McPherson, 1997, Copyright Elsevier, reproduced with permission).

2.7 Minor components of starch

2.7.1 Lipids

Starch and lipids are important components that play a vital role in the functional interactions in food systems (Tang & Copeland, 2007). The presence of starch-lipid complexes influence the digestion by reducing the contact between enzyme and substrate and the extent of swelling is less because of increasing hydrophobicity (Svihus, Uhlen, & Harstad, 2005). The amylose-lipid complex forms a coil to helix transition and the lipids pass into the helical cavities resulting in changes in the rheology of starch (Singh, Singh, & Saxena, 2002). It has been shown that the removal of lipids increases resistant starch content (Zhou, Robards, Helliwell, & Blanchard, 2007). Among food starches, non-waxy cereal starches are unusual because they contain significant amounts of monoacyl lipids (Morrison, Law, & Snape, 1993).

Cereal starches such as wheat, barley, rice, maize contain more lipids (0.2 – 0.8%, w/w) than tuber (0.05%), root (0.1%) and legume (less than 0.6%) starches (Hoover & Manuel, 1996; Gunaratne & Hoover, 2002; Debet & Gidley, 2006). Starch and lipids are the important constituents of foods that play vital roles in caloric density, texture, and flavour of foods (Ai, Hasjim, & Jane, 2013). The amount of lipids present in all normal-amylose cereal starches is proportional to the amylose content (Nebesny, Rosicka, & Tkaczyk, 2002). Lipids or surfactants are used in starch-containing foods as modifiers (Cui & Oates, 1999).

Integral lipids in cereal starches are in the form of lysophospholipids and free fatty acids and surface lipids comprise triglycerides, glycolipids, phospholipids and free fatty acids (Tester, Karkalas, & Qi, 2004). The glycolipids present are digalactoside diglyceride and monogalactosyl diglyceride and the major phospholipids include phosphatidyl choline, N-acyl phosphatidyl ethanolamine and N-acyl lysophosphatidyl ethanolamine (Morrison, 1977). Lipids obtained from field peas constitute 2.9% of the seed weight and contain 43.2% neutral lipids, 3.2% glycolipids and 53.6% phospholipids (Hoover, Cloutier, Dalton, & Sosulski, 1988).

Starch-lipid interactions have been studied by various methods such as iodine absorption, enzymatic analysis, X-ray diffraction, differential scanning calorimetry etc. (Eliasson & Kim, 1995). Starch-lipid complexes have several applications in food industry. It is used to decrease stickiness of starchy foods, enhance freeze-thaw stability, to delay bread staling and are also used as crumb softeners in breads (Copeland, Blazek, Salman, & Tang, 2009).

2.7.2 Proteins

Starch accounts for approximately 0.3% starch granule-associated proteins in cereals and less than 0.1% in potato starch (Xian-Zhong & Hamaker, 2002). Starch granules have a protein content of 3g or less/kg and the proportion increases towards the surface of the granule. The size of a large amount of surface proteins range from 5-60 kDa, whereas proteins present in the interior range from 60-150 kDa (Svihus, Uhlen, & Harstad, 2005). Proteins that are associated with starch granules are present on the surfaces (that can be

readily extracted at temperatures below the gelatinization temperature) or in the form of integral proteins (that are extractable near or above the gelatinization temperature) (Ellis, Cochrane, Dale, Duffus, Lynn, Morrison et al., 1998).

The maize granule-associated proteins comprise two classes: the surface-located zeins that can be removed by proteases and the granule-intrinsic proteins that are resistant to protease digestion (Xian-Zhong & Hamaker, 2002). In the case of wheat, softness and hardness of the grain are associated with the presence or absence of a protein called friabilin on the surface. Higher levels of friabilin are present in the starch granules of soft wheats in comparison to hard ones (Darlington, Tecsi, Harris, Griggs, Cantrell, & Shewry, 2000). In pulses, the major proteins present are globulins and albumins. The globulins comprise two major proteins characterized by their sedimentation coefficients (7S and 11S). The 7S and 11S globulins in pea and fababean refer to vicilin and legumin respectively (Gueguen, 1983). Other protein types present in legumes include various enzymes, protease inhibitors and lutins that are referred to as antinutritional compounds (Roy, Boye, & Simpson, 2010). Some minor proteins such as prolamins and glutelins are also found. Pulse proteins are rich in lysine, leucine, aspartic acid, glutamic acid and arginine but lack methionine, cysteine and tryptophan (Boye, Zare, & Pletch, 2010)

2.7.3 Phosphorous

Starches contain small quantities of minerals that include calcium, magnesium, phosphorous, sodium and potassium (Tester, Karkalas, & Qi, 2004). Most cereal starches contain phosphorous (0.02 – 0.06%) in the form of phospholipids, whereas in the tuber

(0.01 – 0.1%) and pulse (green pea, lentils, lima bean, and mung bean) starches (0.004 – 0.007%), phosphorous is present in the form of starch phosphate monoesters (Singh, Singh, Kaur, Sodhi, & Gill, 2003; Lim, Kasemsuwan, & Jane, 1994; Ambigaipalan, Hoover, Donner, Liu, Jaiswal, Chibbar et al., 2011; Gunaratne & Hoover, 2002; Łabanowska, Wesełucha-Birczyńska, Kurdziel, & Puch, 2013). The distinctive properties of potato starch are attributed to its high level of phosphate esters (Karim, Toon, Lee, Ong, Fazilah, & Noda, 2007). In native potato starch, starch phosphate monoesters are present mainly in the amylopectin (Hoover, 2001) and are linked to the O-2, O-3 or O-6 hydroxyl groups (Blennow, Engelsen, Munck, & Møller, 2000). The phosphate groups (60-70%) are bound to the C-6 position of the glucosyl units as monoesters and 30-40% is monoesterified on the C-3 position (Blennow, Bay-Smidt, Olsen, & Møller, 2000). High swelling power of potato starch is probably due to the presence of phosphate groups and the large granular size (Jobling, 2004). The presence of phosphate groups exert a major influence in the rheological properties of starch resulting in clearer gels and higher viscosity and this is beneficial in several industrial applications (Blennow, Bay-Smidt, Wischmann, Olsen, & Møller, 1998).

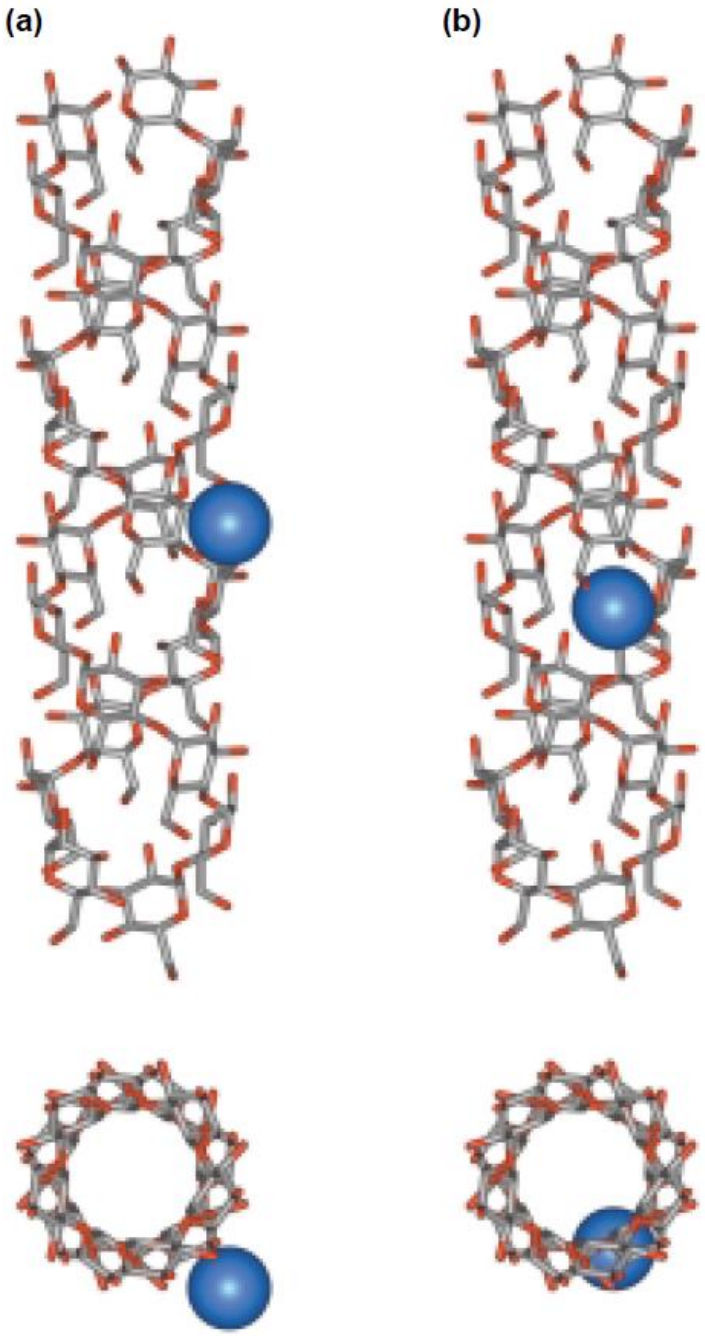

Figure 2.13: A molecular model of phosphorylated starch (crystalline domain). The helices are phosphorylated on the same glucose residue, at the C-3 (a) and C-6 (b) positions (Blennow at al., 2002, Copyright Elsevier, reproduced with permission).

2.8 Disadvantages of native starch

The hydrophilic nature of starch is the main factor restricting the development of starch-based materials (Fang, Fowler, Tomkinson, & Hill, 2002). Though native starch is a good texture stabilizer and regulator in food systems, limitations such as low shear resistance, thermal resistance/decomposition and higher retrogradation rates restrict its application in industries (Ribeiro, do Prado Cordoba, Colman, de Oliveira, Andrade, & Schnitzler, 2014). In order to overcome these drawbacks, native starch is physically, chemically or enzymatically modified to obtain desired properties (Sorokin, Kachkarova-Sorokina, Donzé, Pinel, & Gallezot, 2004). Physical modification of starch by various means such as radiation, heat, shear and moisture is preferred because of the absence of by-products of chemical reagents in the modified starch. Heat moisture treatment and annealing are methods used to alter the physicochemical properties of starch without causing changes to the granular structure (Zavareze & Dias, 2011). Chemical modification is generally carried out through derivatization such as etherification, esterification and crosslinking, oxidation, cationization, grafting and decomposition (Kaur, Ariffin, Bhat, & Karim, 2012). Modified starches exhibit better paste clarity and stability, improved resistance to retrogradation and freeze-thaw stability (Waliszewski, Aparicio, Bello, & Monroy, 2003). Modified starches result in changes in the gelatinization, pasting and retrogradation properties (Singh, Kaur, & McCarthy, 2007) are used as food ingredients (Sweedman, Tizzotti, Schäfer, & Gilbert, 2013).

2.9 Applications of starch

Because of the abundance as a natural biopolymer, starch acts as a very good adsorbent. It is a renewable resource and economically feasible (Ismail, Irani, & Ahmad, 2013). Starch is included in fluid products to improve their viscosity and stability and in semisolid products to enhance their fat and water-holding properties (Hermansson & Svegmark, 1996). It is also used in food industries as a viscosifier, stabilizer, texturizer, binder and for pharmaceutical purposes such as coating, disintegrating, and thickening (Srijunthongsiri, Pradipasena, & Tulyathan, 2014).

Starch is used in bread making, confectionery (Nuwamanya, Baguma, Wembabazi, & Rubaihayo, 2013) and to thicken continuous phase of fluid foods (Chamberlain & Rao, 1999). It is also used as an industrial feedstock and is a source of energy for humans and animals (Morell & Myers, 2005). The gelling properties of starch are useful in controlling the texture and mechanical properties of many foods (Pinto, Vanier, Klein, Zavareze, Elias, Gutkoski et al., 2012).

Native starch, when processed under high pressure and temperature yields a thermoplastic product that can be transformed into injection or blow moulded articles (Funke, Bergthaller, & Lindhauer, 1998). Starch containing a high proportion of amylopectin is used in the food industry to enhance uniformity, texture and also provide freeze-thaw stability in frozen foods (Slattery, Kavakli, & Okita, 2000). Starch is also used as a delivery vehicle that helps in protecting pharmaceutically active proteins from digestion (Jobling, 2004) and is used in cement as an additive to improve the setting time and is

used in paper-making for various purposes (Burrell, 2003). Also, ethanol produced from starch is considered an environmentally friendly option to petroleum based fuels (Hannah & James, 2008). Starch-based biodegradable polymers can be used as medical polymer materials because it offers a host of benefits: good biocompatibility, biodegradability, non-toxicity of degradation products and good mechanical properties (Lu, Xiao, & Xu, 2009). The use of starch in nanotechnology is gaining momentum especially in the area of drug delivery (Rodrigues & Emeje, 2012).

2.10 Starch properties

2.10.1 Granular swelling and amylose leaching

Swelling ability of starch accounts for the important properties such as pasting and rheological behaviors in most starchy food products. When the starch granule is heated in excess water, heat transfer and moisture transfer phenomena occur and the granule swells several times its initial size due to the loss of crystalline order and causes absorption of water inside the granular structure (Lii, Tsai, & Tseng, 1996). Swelling is primarily a property of amylopectin and amylose acts as a dilutant. The swelling pattern is influenced by the magnitude of interactions between the glucan chains within the amorphous and crystalline region and also by the packing arrangement of the glucan chains within the crystalline lamellae (Ratnayake, Hoover, & Warkentin, 2002).

Starch granules are insoluble in cold water and starch normally contains about 20% by weight of water at room temperature. When starch granules are dispersed in water and warmed below the gelatinization temperature, water enters reversibly into the starch structure (Hancock & Tarbet, 2000). Pulse starches exhibit a single stage restricted swelling and low extent of amylose leaching and this is attributed to the strong interactions between starch chains that relax over one temperature and not multiple temperatures. At temperatures below 60°C, no measurable granule swelling or amylose leaching occurs in most pulse starches but a pronounced increase in both swelling and amylase leaching occurs beyond 70°C and this could be correlated to the high amylose content of pulse starches which causes the tight packing of amylose chains within the

amorphous domains of the granule. This result in strong interactions between adjacent amylose chains and therefore a high input of thermal energy is required to disrupt the amylose chain interactions (Hoover, Hughes, Chung, & Liu, 2010).

2.10.2 Gelatinization

Native starches are insoluble in cold water. When starch granules are heated in excess water, it undergoes an order to disorder phase transition at a certain temperature interval referred to as the gelatinization temperature range. As a result of this, substantial rheological changes take place in the starch suspension during heating. The starch granules imbibe water, swell to several times their original size and results in the leaching of amylose, the low molecular weight components of the starch granules (Eliasson, 1986). Finally, the crystallites are disrupted and there is a total loss of crystallinity (Karapantsios, Sakonidou, & Raphaelides, 2002). Starch gelatinization in water is thus the breakdown of intermolecular association between amylose and amylopectin molecules by the application of heat (Tako, Tamaki, Teruya, & Takeda, 2014). This property is vital in contributing to starch functionality and thus used in food industries (Bogracheva, Morris, Ring, & Hedley, 1998). Gelatinization causes several changes in the physical, chemical and biological properties of starch (Shetty, Lineback, & Seib, 1974). Though the gelatinization process is readily apparent, a number of methods used for the determination has made it hard to formulate a precise definition (Zobel, Young, & Rocca, 1988).

Gelatinization is one of the unique properties of starch granules and it is generally accepted that water first enters the amorphous region and initiate swelling that result in the loss of birefringence as the temperature is increased. As a result of further increase in temperature, thermal motion and solvation causes decreasing order and rupture of crystalline regions with uncoiling of double helices (Lineback, 1986). Formation of a

viscous solution or a gel is primarily dependent on the starch/water ratio. However, there are other processing parameters such as temperature and heating rate influencing this process (Sakonidou, Karapantsios, & Raphaelides, 2003). A clear endothermic peak that is visible in the temperature region between 54 and 73°C for various starches was defined as the gelatinization temperature (Yu & Christie, 2001). Thus, starch phase transitions are three stage processes in which the starch granules absorb water increasing the starch polymer mobility in amorphous regions, formation of new intermolecular interactions and an increase in hydrothermal effects take place causing the disruption of overall granule structure (Ratnayake & Jackson, 2007).

Gelatinized starches have a number of industrial applications apart from its non-food uses such as in drilling oil wells, sizing textiles, paper manufacture, briquetting charcoal, etc. Gelatinized starches are used in foods as a thickener. The gelatinization of starch also influences the characteristics and quality of food, elasticity and softness of paste products, digestibility and palatability (Chiang & Johnson, 1977). Gelatinization plays a significant role in several food processing operations and hence many analytical techniques such as light microscopy, electron microscopy, viscometry, x-ray diffraction, nuclear magnetic resonance, calorimetry, laser light scattering etc. have been used to estimate quantitatively the amount of gelatinized starch in processed foods (Biliaderis, 1991). It is also important in analysis and design of food processes such as extrusion, aseptic processing and sterilization (Wang & Sastry, 1997).

DSC measures the onset (T_o), midpoint (T_p), conclusion (T_c) temperatures and the enthalpy (ΔH) of gelatinization. DSC parameters are dependent on the molecular

architecture of the crystalline region, that relates to the distribution of the amylopectin short chains (DP 6-11) and not by the proportion of crystalline region that corresponds to the amylose to amylopectin ratio. A low T_o, T_p, T_c and ΔH indicate the presence of abundant short amylopectin chains (Ratnayake, Hoover, & Warkentin, 2002). Gelatinization temperature is positively correlated with the amylopectin long branch chains as it could form longer double helices and thus would require higher temperatures for complete dissociation. Song and Jane (2000) reported higher onset temperature of gelatinization for high amylose starches than waxy and normal maize starch because of the longer branch chain lengths. The presence of phosphate monoesters are reported to cause a decrease in gelatinization temperatures. The ratio of amylose to amylopectin is also an important factor influencing the gelatinization temperature of starches. Waxy starches exhibited a broader gelatinization temperature and higher enthalpy in comparison with normal starches which suggests that the structure of amylopectin mainly determines the gelatinization temperatures (Emmambux & Taylor, 2013).

2.10.3 Retrogradation

When starch granules are heated in the presence of excess water (above their gelatinization temperature), they undergo irreversible swelling and result in the leaching of amylose into the solution. This suspension will form an elastic gel on cooling and the molecular interaction that take place after cooling is called retrogradation, which is time and temperature dependent (Ratnayake, Hoover, & Warkentin, 2002).

Starch retrogradation occurs in three steps: nucleation (formation of crystal nuclei), propagation (crystal growth from the nuclei formed during nucleation) and maturation (Ambigaipalan, Hoover, Donner, & Liu, 2013). Starch retrogradation is not desirable because it is responsible for the staling of bread and other starch-rich foods, resulting in reduced shelf-life and consumer acceptance but is found to be useful in the production of breakfast cereals, parboiled rice, in the manufacture of croutons and breadcrumbs (Ottenhof & Farhat, 2004) due to the modification of structural and mechanical properties (Wang, Li, Copeland, Niu, & Wang, 2015).

Retrogradation occurs in two stages: the recrystallization of amylopectin which is a slower process and the gelation of solubilized amylose that proceeds at a faster rate. The turbidity increases during the early stage of storage and this indicates the network formation between amylose chains leached out of the granules during gelatinization (Fukuzawa, Ogawa, Nakagawa, & Adachi, 2016).

Starch molecules in gel associate on aging, resulting in precipitation, gelation and changes in consistency. Crystallites start to develop and this is followed by increases in

rigidity and phase transition between polymer and solvent (Karim, Norziah, & Seow, 2000). Retrogradation involves increase in the degree of crystallinity and gel firmness, exudation of water and the appearance of "B" type X-ray pattern. The legume starches are susceptible to retrogradation and syneresis and so are not suitable for products that call for low-temperature storage. Retrogradation is occasionally used in modifying the structural, mechanical and organoleptic properties of starch-based products (Liu, Yu, Chen, & Li, 2007).

Though both amylose and amylopectin have the ability to retrograde, the long-term quality changes in foods is attributed to the amylopectin component (Sandhu & Singh, 2007). The retrogradation of starch results in a reduction in the quality of starchy foods and also negatively impacting their textural properties. Many methods such as X-ray diffraction, differential scanning calorimetry etc. have been used for determining the degree of retrogradation (Fukuzawa, Ogawa, Nakagawa, & Adachi, 2016) and so it becomes difficult to measure the rate and extent of retrogradation. Each of these methods measures a different property of retrogradation. For example, syneresis data and turbidity measurements provide information on the amylose and amylopectin crystallization. On the other hand, DSC and NMR measures amylopectin crystallization and changes in water mobility during retrogradation (Hoover, Hughes, Chung, & Liu, 2010).

DSC has been used in investigating gelatinization in water-starch systems (Nakazawa, Noguchi, Takahashi, & Takada, 1984). DSC has been used for studying starch retrogradation for the following reasons: 1. It can be used for a wide range of water content, 2. Direct determination of the heat of re-gelatinization after retrogradation is

possible, 3. No change in water content because of perfect sealing of the sample cells, 4. The technique is not time consuming (Nakazawa, Noguchi, Takahashi, & Takada, 1985).

Retrogradation occurs because the gelatinized starch is supercooled and maintained below its melting temperature and hence the potential towards thermodynamic equilibrium during storage causes reordering and crystallization (Lionetto, Maffezzoli, Ottenhof, Farhat, & Mitchell, 2006). The primary reason for the deterioration of desirable sensory qualities of starch containing products is the retrogradation of starch during storage. Methods used for retarding retrogradation include enzyme treatment, addition of oligosaccharides and lipids (Yao, Zhang, & Ding, 2003).

Lipids and short amylopectin chains with DP 6-9 have also been reported to reduce retrogradation (Fredriksson, Silverio, Andersson, Eliasson, & Åman, 1998). Generally, cereal amylopectin retrograde to a lesser extent in comparison to pea and potato amylopectin due to the presence of shorter average chain lengths in cereal amylopectin (Silverio, Fredriksson, Andersson, Eliasson, & Åman, 2000). Sugars such as glucose, fructose and maltose have been reported to inhibit the rice and potato starch retrogradation. Hydrogen bonding between the starch chains that lead to crystallization can be modified by saccharides that result in reduction in the rate of retrogradation (Durán, León, Barber, & de Barber, 2001). Because of the higher amylose content and/or differences in molecular structure, pulse starches retrograde to a higher extent in comparison with cereal and tuber starches (Hoover, Hughes, Chung, & Liu, 2010).

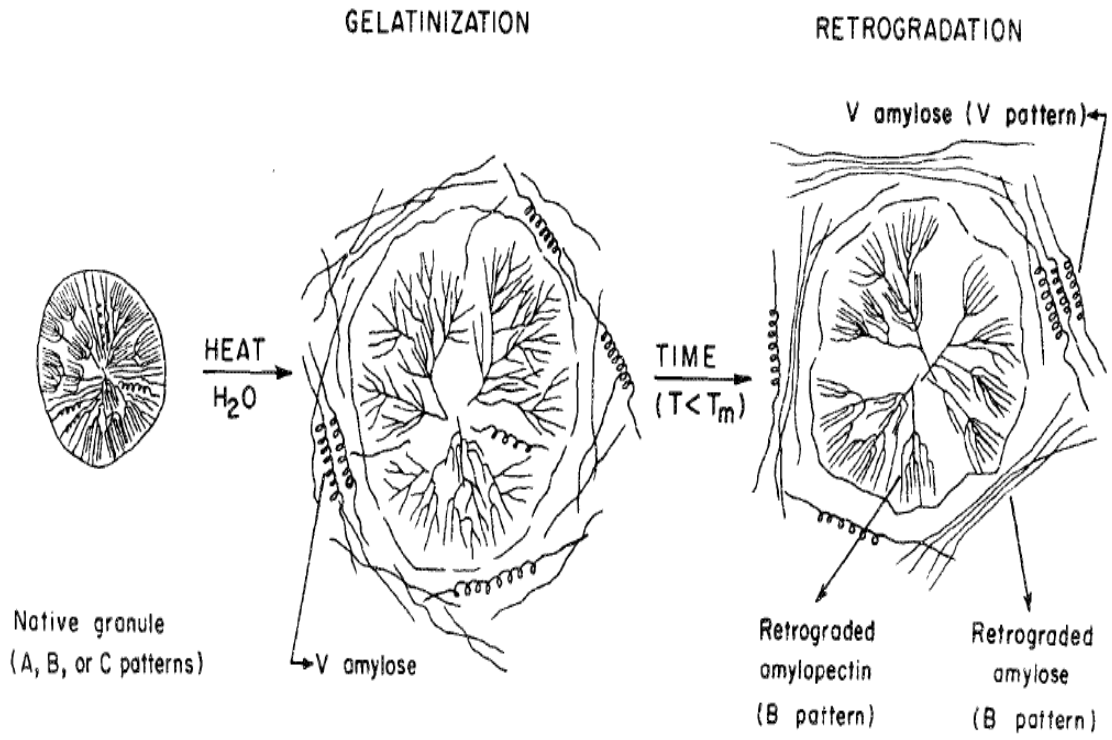

Figure 2.14 : A schematic representation of the processes and structures observed during heating and storage of aqueous suspensions of granular starch (Biliaderis, 1991, reproduced with permission from NRC Research Press)

2.10.4 Pasting

Pasting characteristics were first described by Caesar in 1932 using a consistometer. The Brabender amylograph available during the 1930s was used in the industry for characterisation of starch containing products (Deffenbaugh & Walker, 1989). The term pasting refers to the changes that occur after gelatinization. The pasting properties of a starch suspension can be monitored with a rapid visco analyzer (RVA) (Waterschoot, Gomand, Fierens, & Delcour, 2015). Pasting properties are influenced by starch granule size, amylose and lipid contents and structure of amylopectin (Ao & Jane, 2007).

Starch granules are insoluble in water below the onset temperature of gelatinization (usually below 50°C). When starch granules are heated in the presence of water above the gelatinization temperature, the granules absorb water and swell to many times their original size resulting in the leaching of amylose and an increase in viscosity. The temperature at the beginning of rise in viscosity is considered as the starting point of gelatinization and is referred to as the pasting temperature (Pongsawatmanit, Thanasukarn, & Ikeda, 2002). The highest viscosity reached during the heating cycle is the peak viscosity (Vamadevan & Bertoft, 2015). Peak viscosity reflects the water binding capacity and the extent to which the starch granules are disintegrated and is related to the final product quality (Gani, Wani, Masoodi, & Salim, 2013). With increasing amylose content, peak viscosity of the starch paste decreases (Blazek & Copeland, 2008). The peak viscosity decreases as the granules rupture due to continuous shear and starch molecules are dispersed in aqueous phase (Copeland, Blazek, Salman, & Tang, 2009). This is referred as breakdown and the breakdown value indicates the paste

stability (Joshi, Aldred, McKnight, Panozzo, Kasapis, Adhikari et al., 2013) and the differences in breakdown are due to differences in rigidity/fragility of the swollen granules (Han & Hamaker, 2001). As the starch paste cools, molecules leached out begin to reassociate in an ordered structure. (Jacobs & Delcour, 1998). The extent of reassociation depends on several factors such as amylose content, length of amylose molecules and the state of dispersion of amylose chains (Mishra & Rai, 2006). Amylose molecules, because of their linear structure reassociate more easily and is one factor contributing to the occurrence of setback (Rupollo, Vanier, Zavareze, Oliveira, Pereira, Paraginski et al., 2011).

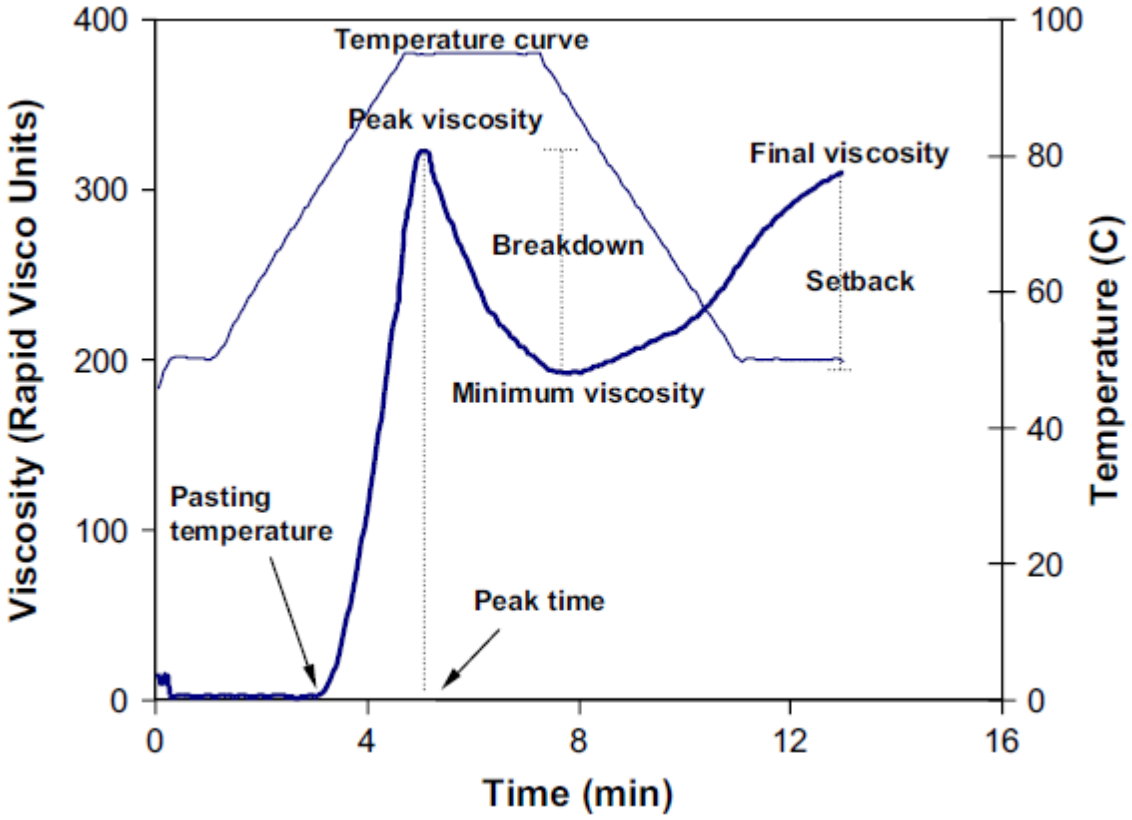

Figure 2.15: Typical RVA profile of rice starch (Copeland et al., 2009, Copyright Elsevier, reproduced with permission)

2.10.5 Acid hydrolysis

Native starches cannot be utilized for specific industrial applications owing to its functional limitations such as low resistance to shear stress, high tendency towards retrogradation and poor thermal properties (Hermansson & Svegmark, 1996). However, these limitations can be overcome by physical (heat-moisture treatment, annealing, pre-gelatinization, radiation) and chemical (crosslinking, acid hydrolysis, substitution, oxidation/bleaching) modification methods (BeMiller & Huber, 2010). The physical modification technique is generally preferred because of the absence of by-products of chemical reagents in the modified starch (Zavareze & Dias, 2011).

Acid hydrolysis provides an understanding of the inner structure of starch granules as it could result in changes in the morphological, crystalline, gelatinization and viscoelastic properties of starch (Jiping, Shujun, Jinglin, Hongyan, Jiugao, & Wenyuan, 2007). Acid hydrolysis involves the treatment of starch granules with dilute aqueous or alcoholic solutions of hydrochloric or sulphuric acid below the gelatinization temperature for different time periods and when the desired degree of hydrolysis is attained, the resultant modified starch is recovered by neutralizing with alkali followed by washing and subsequent drying (Wang & Copeland, 2015).

The hydrolysis of cereal, legume and tuber starches follows a two stage pattern: rapid hydrolysis of the amorphous region and the slow hydrolysis of crystalline regions (Singh, Sodhi, & Singh, 2009). Two hypotheses have been proposed for the slower hydrolysis of crystalline region. Firstly, the dense packing of starch chains within the starch crystallites

prevents the penetration of H_3O^+ into the regions. Secondly, the chair to half- chair transformation that is necessary for the hydrolysis of the glucosidic bond occurs slowly due to the immobilization of the sugar conformation within the starch crystallites (Jayakody & Hoover, 2002).

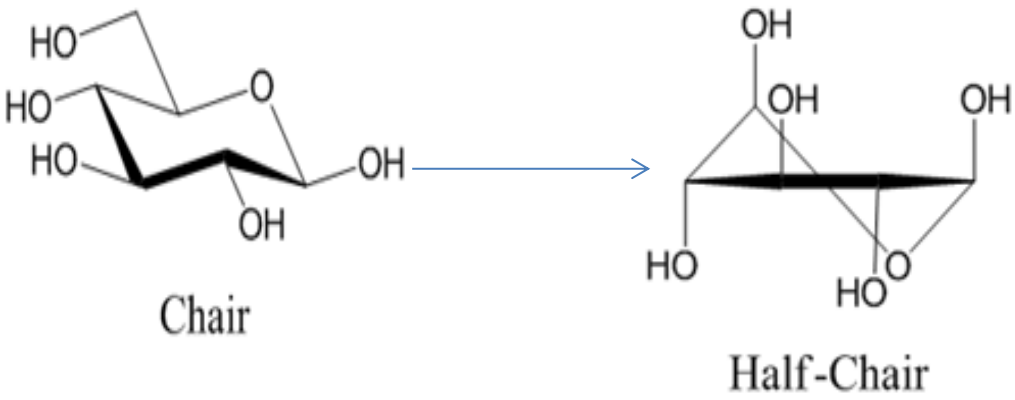

Figure 2.16: Chair to half-chair conformation of glucose molecule

2.10.6 Enzyme hydrolysis

Legume starches are better substrates for analyzing the structural factors contributing to α-amylolysis than cereal and tuber starches because of the absence of pores on the granular surface, lesser quantities of phosphate monoesters, trace amounts of bound lipids and granule size uniformity (Zhou, Hoover, & Liu, 2004). Starch hydrolysis involves an enzyme in solution reacting on a solid substrate and thus the critical kinetic parameters are the surface area that is accessible to the enzyme and the extent of enzyme adsorption onto the surface (Hoover & Zhou, 2003). Therefore, the steps involved in the enzymatic reaction are the diffusion to the solid surface, adsorption of the enzyme and the occurrence of catalysis (Uthumporn, Zaidul, & Karim, 2010). Native starch is digested slowly in comparison with the gelatinized starch because the accessibility of the enzyme to the substrate in processed starch is greater and also the crystallinity is disrupted. Furthermore, there are no restrictions by α-glucan associations (Blazek & Gilbert, 2010). The smaller granules hydrolyze faster because of their large surface area per unit mass that facilitates diffusion and adsorption of enzymes and increasing the catalytic action in comparison with large granules (Dhital, Shrestha, & Gidley, 2010).

Starch hydrolysis by α-amylase depends on several features such as granule size, phosphorous content, presence of amylose-lipid complexes, starch crystallinity and porosity (Asare, Jaiswal, Maley, Båga, Sammynaiken, Rossnagel et al., 2011). Amylolytic enzymes are not capable of bypassing the phosphorylated glucosyl residue and so digestion of potato starch (that contains covalently-bound phosphate groups) with amylase results in the release of phosphoryl oligosaccharides (Absar, Zaidul, Takigawa,

Hashimoto, Matsuura-Endo, Yamauchi et al., 2009). Thus, the presence of higher phosphorous content in potato starch may be associated with reduced digestibility of both gelatinized and native starch (Noda, Takigawa, Matsuura-Endo, Suzuki, Hashimoto, Kottearachchi et al., 2008). Amylose-lipid complexes restrict the susceptibility of amylose to amylolysis. The competitive mechanism is believed to exist between amylose retrogradation and amylose-lipid complex formation, wherein the crystallization of amylose-lipid complexes is favoured that prevents amylose leaching during gelatinization and restricts the swelling of starch granules when heated in the presence of water (Cui & Oates, 1999). The porosity of starch granules has been reported to influence the extent of enzyme hydrolysis as the presence of pores, channels and cavities increase the surface available for the enzymatic reactions. Pores provide access for initial enzyme attack that causes the enzyme to penetrate to the granule interior (Sujka & Jamroz, 2007). Double helices that associate to form crystalline regions resist enzyme hydrolysis and it has been reported that B and C-type starches are more resistant to hydrolysis than A-type (Tester, Qi, & Karkalas, 2006).

Starch digestibility is influenced by some unique properties of legumes such as high content of viscous soluble dietary fibre, various antinutrients and high amylose/amylopectin ratios (Sandhu & Lim, 2008). It is reported that amylolysis occurs along furrows initially and then pass through pores that increases in size (Svihus, Uhlen, & Harstad, 2005). Starch with ‘A’ type diffraction pattern is more susceptible to amylolysis because the branch points in ‘A’ type starch are scattered in both amorphous and crystalline regions. As a result of this, numerous short A chains are present in the

crystalline regions forming an inferior crystalline structure that lead to weak points and thus readily attacked by α-amylase. On the other hand, in 'B' type starches, amorphous region has more branch points and fewer short branch chains that results in a superior crystalline structure in comparison to 'A' type starches and thus more resistant to amylolysis (Srichuwong, Sunarti, Mishima, Isono, & Hisamatsu, 2005).

Native starches are hydrolyzed to a lower extent than gelatinized starch. During the enzyme-catalyzed hydrolysis of α(1→4) glucosidic bonds, the ring distortion from chair to half-chair conformation decreases the enthalpy of activation and increases the susceptibility of glucosyl residues to nucleophilic attack by functional groups on the enzyme and by water (Oates, 1997).

Enzyme hydrolysis has multiple benefits in comparison with the acid that was traditionally used to hydrolyze starch that resulted in the formation of low molecular weight dextrins and glucose. The specificity of enzyme results in the sugar syrup production with well-defined properties and the enzymatic hydrolysis is milder that causes less 'browning' and a few side reactions (Nigam & Singh, 1995). The enzyme hydrolysis also results in changes in functional properties of starch such as gelatinization temperatures, gel formation and paste viscosity (Rocha, Carneiro, & Franco, 2010).

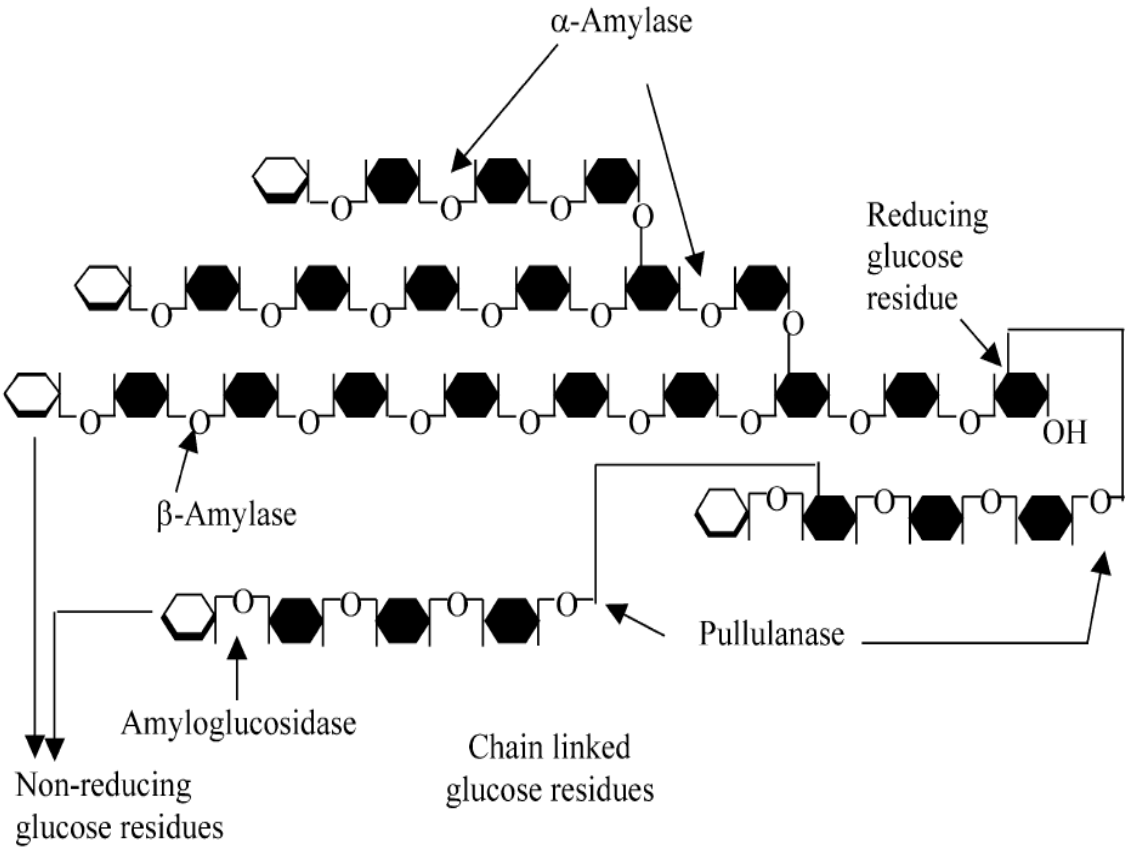

Figure 2.17: Action pattern of starch-degrading enzymes (Tharanathan, R., & Mahadevamma, S., 2003, Copyright Elsevier, reproduced with permission).

2.10.7 Starch nutritional fractions:

Starch is classified into RDS (rapidly digestible starch), SDS (slowly digestible starch) and RS (resistant starch) based on the extent of glucose release and its absorption in the gastrointestinal tract (Zhang, Ao, & Hamaker, 2006). If the amount of RDS present is high, SDS and RS would be low (Zhang & Hamaker, 2009). The rate of glucose release from foods play a vital role in human health by aiding to control the blood glucose levels and to provide good energy absorption (Zhang, Venkatachalam, & Hamaker, 2006). The concept of glycaemic index (GI) was proposed to classify foods based on their postprandial blood glucose response (Jenkins, Wolever, Taylor, Barker, Fielden, Baldwin et al., 1981). The GI is defined as "the postprandial incremental glycemic area after a test meal that is expressed as a percentage of the corresponding area after an equi-carbohydrate portion of a reference food such as white bread" (Goñi, Garcia-Alonso, & Saura-Calixto, 1997).

RDS is the fraction of starch granules that cause a rapid elevation in blood glucose concentration after carbohydrate ingestion. SDS is the portion of starch that is digested slowly but completely in the small intestine and the part of starch that escapes digestion in the small intestine and is fermented in the colon as dietary fiber is referred to as resistant starch (Dona, Pages, Gilbert, & Kuchel, 2010). One of the primary aims to manage diabetes is to decrease meal-associated hyperglycaemia. Intake of slowly digestible starch provides a host of benefits such as reduced postprandial glucose peaks, improved lipid response, lesser concentration of glycosylated haemoglobin and fructosamine and better insulin sensitivity (Lehmann & Robin, 2007). SDS is widely used

in edible processed food products, nutritional supplements and drug preparations. SDS materials used in food processing exhibit a low thermal stability. It is also used as a biomacromolecule film-former for pharmaceutical coatings to allow complete release in the small intestine (Miao, Jiang, Cui, Zhang, & Jin, 2015).

RS content in foods is influenced by the amylose/amylopectin ratio, degree of gelatinization, thermal treatments and cooling/storage (Goñi, Garcia-Diz, Mañas, & Saura-Calixto, 1996). Several factors contribute to the amount of RS in starch-rich foods: the source, ripeness, processing, preparation and storage (Raben, Tagliabue, Christensen, Madsen, Holst, & Astrup, 1994). Due to the presence of surplus quantities of salivary and pancreatic α-amylase, starch was believed to be hydrolyzed completely and absorbed from the small intestine but it was later observed that a portion of starch resists water dispersion and pancreatic amylase hydrolysis. Starch that does not breakdown in the small intestine will enter the large intestine where it acts in the same manner to the unabsorbed non-starch polysaccharides of dietary fiber (Tomlin & Read, 1990). Resistant starch has thus been defined as 'the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals' (Asp, 1992).

The purpose of incorporating an ingredient high in RS is to combine physical, nutritional functionalities and processing stability (Thompson, 2000). A diet containing a high proportion of dietary fiber and RS has been found to significantly increase the weight of pancreas, liver and intestine in rats (Perera, Meda, & Tyler, 2010). Resistant starch forms only a small proportion of the total calories in diets and so its inability to be digested and converted to glucose makes little impact to the overall energy intake (Johnson & Gee,

1996). Resistant starch can be used for the development of functional foods by employing processing techniques to increase the resistant starch level in foods, identifying foods rich in resistant starch and processing them to be palatable to consumers (Niba, 2002). Resistant starch undergoes fermentation upon the activity of large bowel microflora that results in the production of methanol, hydrogen and short chain fatty acids which decrease pH and stimulates the formation of beneficial groups of bacteria and eliminating pathogenic microorganisms (Leszczyñski, 2004).

Resistant starch is classified into several types. Type 1 refers to the physically trapped starch that are found locked in the plant cell and present in partly milled grains, seeds and legumes (Eerlingen & Delcour, 1995). This refers to starch granules that are physically inaccessible to digestion and is released when food is ground, chewed or milled (Muir, Young, & O'Dea, 1994). RS2 are native, uncooked granules of starch (Fuentes-Zaragoza, Riquelme-Navarrete, Sánchez-Zapata, & Pérez-Álvarez, 2010). RS2 refers to starch that exists in a granular form and resistant to enzyme digestion and is found in raw potato and green banana (Raigond, Ezekiel, & Raigond, 2015). RS3 indicates retrograded starch and the starch granules are completely hydrated in the formation of RS3. Amylose leaches from the granules into the solution and the polymer chains begin to reassociate as double helices upon cooling (Sajilata, Singhal, & Kulkarni, 2006). Significance of RS3 is attributed to its thermal stability as it remains stable in most cooking operations and thus serving as an ingredient in a number of conventional foods (Haralampu, 2000). In addition to the naturally occurring resistant starch types, a fourth category includes chemically modified starches that resist enzyme hydrolysis to a certain degree.

Modification of starch by crosslinking, esterification, etc. is performed to extend the usefulness of starch in food processing, transportation and storage (Brown, 1996).

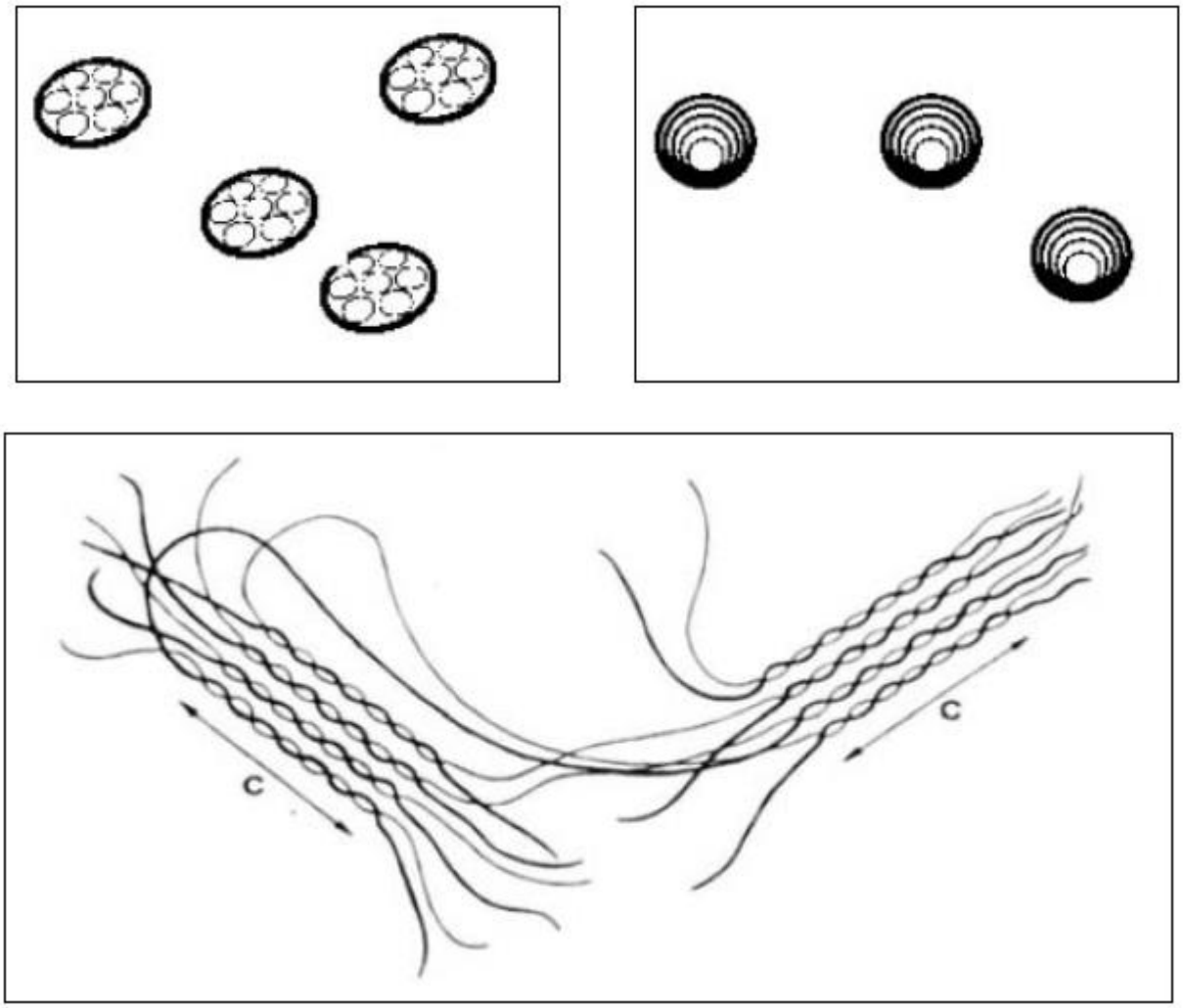

Figure 2.18: Structure of resistant starch A) Type 1 resistant starch B) Type 2 resistant starch C) Type 3 resistant starch formed in aqueous amylose solutions. (Sajilata et al., 2006, reproduced with permission from John Wiley and Sons).

Chapter 3

Materials and methods

3.1 Materials

Field pea (*Pisum sativum*) cultivars [CDC Golden (CDCG), Abarth (ABAR), CDC Patrick (CDCP), CDC Amarillo (CDCA)] grown (during the 2012 season) at two different locations (Rosthern and Meathpark) in Saskatchewan, Canada were obtained from the Crop Development Center, University of Saskatchewan, Saskatoon, Canada. The locations at which the field peas were grown differed only marginally with respect to rainfall, growing season, temperature and soil conditions. Pancreatin from porcine pancreas (Cat. No. P-1625, activity 3 x USP/g) was purchased from Sigma Chemical (St Louis, MO). Amyloglucosidase (EC 3.2.1.3., 3300 U/ml) and glucose oxidase-peroxidase assay kit (cat. no. K-GLUC) were purchased from Megazyme International Ireland Ltd (Bray, Ireland). All chemicals and solvents were of ACS certified grade.

3.2 Methods

3.2.1 Starch isolation

Starch was extracted from the pulse seeds using the wet milling procedure of Hoover and Sosulski (1985). Pulse seeds (50g) were steeped in 250 ml of deionized water containing 0.01% (w/v) sodium metabisulfite for 36 hours at 40^{o}C. The swollen seeds were dehulled by pressing the endosperm out of the softened hull and then thoroughly washed with deionized water. The softened seeds were wet milled (1:3) with sufficient water using

Waring commercial blender (Dynamics Corporation of America, Greenwich, Connecticut, USA) for 3 minutes and then screened through cheese cloth. This step was repeated two more times and pooled the filtrates. The filtrate was then passed through a 210µm polypropylene sieve followed by a 70µm nylon sieve and allowed to sediment for 18 hours at room temperature. The supernatant was decanted and an excess of 0.2% sodium hydroxide was added to the sediment at room temperature and this step was repeated many times until a white starch was obtained. The resulting starch was washed with deionized water and passed through a 70µm nylon sieve. The solution was then neutralized to pH 7.0 with 0.2% hydrochloric acid and filtered on a Buchner funnel using Whatman No.4 filter paper and thoroughly washed on the filter with distilled water. The resulting filter cake was oven dried overnight at 40°C and the dried starch was ground passed through a 60-mesh screen.

3.2.2 Starch damage

Starch damage was estimated by enzyme digestion procedure according to the AACC (2000) method. One gram of starch (dry basis) was measured and added to a 125ml Erylenmeyer flask and 50mg fungal amylase from *Aspergillus oryzae* (157 units/mg protein) was added. Acetate buffer (45 ml) was brought to 30°C in a water bath and then added to the Erylenmeyer flask and the contents were mixed with a glass rod to obtain a uniform suspension before incubating into the water bath at 30°C. The enzyme action was terminated at the end of 15 minutes by the addition of 3ml of H_2SO_4 and 2 ml of sodium tungstate solution. Contents were mixed well and allowed to stand for 2 minutes. The solution was then filtered through Whatman No 4 filter paper. First 8-10 drops of filtrate

were discarded and the determination of reducing sugar was carried out by the procedure outlined by Bruner (1964).

3.2.3 Chemical composition

3.2.3.1 Moisture content

The moisture content of the starch samples were determined by the AACC (2000) method. Empty moisture pans and lids were dried for 1h at 130°C in an oven and then cooled in a desiccator prior to analysis. The moisture pans were pre-weighed and 5g of starch samples were added and dried in an air-forced oven at 130°C for 1h. Moisture pans were placed in the oven with the lids placed under the pans and then the samples were removed, covered with lids and transferred to a desiccator. Weights of the sample were measured after they reached the room temperature. Three replicates were used for the analysis and the moisture content was calculated by the following equation,

$$\text{Moisture (\%)} = \frac{W1-W2}{W1-W0} \text{ X } 100 \text{.....(EQ 3.1)}$$

where,

W_o – weight of the tare moisture pan and lid (g),

W_1 – weight of sample, moisture pan and lid before drying (g), and

W_2 – weight of sample in the pan after drying (g)

3.2.3.2 Nitrogen content

The nitrogen content of native starches was estimated by Micro Kjeldahl method (AACC, 2000). The samples (0.3g, db) were measured on nitrogen-free papers and placed in the digestion tubes of Büchi 430 (Büchi Laboratoriums-Technik AG, Flawill/Schweiz) digester. The catalyst (2 Kjeltabs M pellets [Fisher Scientific, Fair Lawn, NJ, USA]) and 20 ml of concentrated sulphuric acid were added. The samples were digested until a clear yellow solution was visible which was then cooled, diluted with 50ml of distilled water. One hundred milliliters of 40% (w/w) sodium hydroxide were added and the released ammonia was steam distilled into 50 ml of 4% of H_3BO_3 containing 12 drops of end point indicator (N-point indicator, Sigma Chemical Co., St. Louis, MO, USA) using a Büchi 321 distillation unit 150ml of distillate was collected. By titrating against 0.025M sulphuric acid, the amount of ammonia in the distillate was determined and the nitrogen content was calculated using the equation,

$$\text{Nitrogen (\%)} = \frac{(V1-V2)\ X\ N\ X\ 14.0067}{W}\ X\ 100 \quad \text{.....(EQ 3.2)}$$

where

V_1 – volume (ml) of H_2SO_4 to titrate sample,

V_2 – volume (ml) of H_2SO_4 to titrate blank,

N – normality of H_2SO_4, and

W – sample weight (g/db)

3.2.3.3 Apparent amylose content

Amylose content of starches was determined by the colorimetric method of Hoover and Ratnayake (2004). Starch (20 mg/db) was weighed in a round bottom screw cap tube and solubilized by adding dimethylsulfoxide (90% DMSO, 8 ml). The contents were vortexed and heated in a water bath for 15 min at 85°C with intermittent mixing. The tubes were then allowed to cool at room temperature which was then diluted to 25ml in a volumetric flask. 1ml of the diluted solution was mixed with 40ml of water and 5 ml of I_2/KI solution [0.0025M I_2 and 0.0065M KI mixture] and vortexed. The final volume was brought to 50ml in a volumetric flask. The contents were allowed to stand in the dark for 15 minutes at room temperature. The absorbance was measured at 600nm using a UV-visible spectrophotometer. The amylose content of the samples was calculated using a standard curve constructed using the pure amylose and amylopectin mixtures, over the range 0% to 100% (Appendix I).

3.2.3.4 Lipid content

3.2.3.4.1 Surface lipid

Surface lipids of the native starch sample were estimated by the procedure outlined by Vasanthan and Hoover (1992). The surface lipids were extracted at ambient temperature (25 - 27°C) by the addition of 100ml of chloroform/methanol (2:1, v/v) to the starch sample (5g, db). The contents were thoroughly mixed for 1 hour and the solution was filtered into a 250 ml round bottomed flask. The residue was washed out with chloroform/methanol solution. The lipid solvent mixture was evaporated to dryness using

a rotary evaporator (Rotovapor-R110, Büchi Laboratorimus-Technik AG, Flawill/Schweiz, Switzerland). Purification of the crude lipid extracts was carried out by the method of Bligh and Dyer (1959) before quantification.

3.2.3.4.2 Bound lipid

Bound lipid content of starch samples was estimated using the method of Vasanthan and Hoover (1992). The residues from the chloroform/methanol extractions were refluxed with n-propanol/water (3:1, v/v) in a Soxhlet apparatus at 85°C for 7h. The solvent was evaporated to dryness using a rotary evaporator. The crude lipid residue was purified by the method of Bligh and Dyer (1959) before quantification.

3.2.3.4.3 Crude lipid purification

Bligh and Dyer (1959) method was used for the extraction of crude lipid extracts using a separatory funnel with chloroform/methanol/water. The round bottomed flask with crude lipid extract was washed with chloroform/methanol/water (1:2:0.8, v/v/v) respectively. The contents were then mixed and then transferred to separatory funnel. Chloroform and water was then added to form a biphasic layer. The heavy chloroform layer in the bottom of the separatory funnel that contained the purified lipid was withdrawn into a pre-weighted round bottomed flask and then evaporated to dryness in the rotary evaporator. The round bottomed flask containing the purified lipid was removed and dried at 60°C in an air forced oven. The dried lipids were cooled to room temperature in a desiccator and weighed. Lipid content was estimated using the formula:

Lipid (%) = $\frac{W2-W1}{Wo}$ X 100……(EQ 3.3)

where

W_o – sample weight (g/db)

W_1 – weight of tare round bottom flask (g)

W_2 – weight of round bottomed flask and lipid after drying (g)

3.2.4 Granule morphology and particle size distribution

3.2.4.1 Starch granule size distribution

Granule size analysis was carried out using a Laser particle sizer (Fritsch NanoTec "analysette 22", Idar-Oberstein, Germany). The starch samples were suspended in water around 7% (w/w). Particle size is defined in terms of 10^{th} percentile [d(0.1)], median [d(0.5)], 90^{th} percentile [d(0.9)] and surface weighted mean [D(3,2)]. The average granule size was expressed as the surface weighted mean value, i.e. the diameter of a sphere that has the same volume/surface area ratio. This was used to calculate the specific surface area (m^2/g) assuming spherical granules of uniform density (1500 kg/m^3).

3.2.4.2 Light microscopy

Pulse starch suspensions (water/glycerol 50:50, v/v) were observed under bright field (magnification 400x) and crossed polarised light (magnification 200x) using a binocular microscope (Nikon microscope, Eclipse 80i, Nikon INC, Melville, NY, USA) equipped

with a real time viewing (Q-capture PRO^{TM}). A QImaging digital camera (QICAM fast 1394, BC, Canada) was used for image capture.

3.2.4.3 Scanning electron microscopy (SEM)

Granular morphologies of starches were examined using a FEI MLA 650 FEG (Brisbane, Australia) scanning electron microscope at an accelerating potential of 20 kV. The dried starch samples were thinly spread onto circular aluminum stubs with double–sided carbon adhesive tape and then carbon coated in a vacuum chamber.

3.2.5 Starch structure

3.2.5.1 Determination of amylopectin chain length distribution by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

The amylopectin branch chain length distribution was determined by isoamylase debranching of whole starch followed by HPAEC-PAD (Liu et al., 2007). Starch was dispersed in 2 ml of 90% DMSO (5 mg/ml) and subjected to stirring in a boiling water bath for 20 minutes. Upon cooling, 6 ml of methanol was added and vortexed. The tube was then placed in an ice bath for 30 minutes. The pellet was obtained by centrifugation (1000 x g for 12 min) and dispersed in 2 ml of 50mM sodium acetate buffer (pH 3.5) by stirring in a boiling water bath for 20 minutes. 5 µl of isoamylase was added (EN102, 68,000 U/mg protein, Hayashibara Biochemi cals Laboratories Inc., Okayama, Japan) following the equilibration of tube at 37°C. The sample was incubated with slow stirring for 22h at 37°C and the enzyme was inactivated by boiling for 10 minutes. An aliquot

(200 µl) of the cooled debranched sample was diluted with 2 ml of 150 mM NaOH and the sample was filtered in a 0.45 µm nylon syringe filter which was then injected into the HPAEC-PAD system using 50 µl loop. The HPAEC-PAD system consists of a Dionex DX 600 equipped with an ED50 electrochemical detector with a gold working electrode, GP50 gradient pump, LC30 chromatography oven and AS40 automated sampler (Dionex Corporation, Sunnyvale, CA, USA). The standard triple potential waveform was used with the following periods and pulse potentials: T1 = 0.40s, with 0.20s sampling time, E1 = 0.05V; T2 = 0.20s, E2= 0.75V; T3= 0.40s, E3 = -0.15V. Data were obtained using Chromeleon software, version 6.50 (Dionex Corporation, Sunnyvale, CA, USA). The weight fraction of DP 6-12, 13-24, 25-36 and 37-58 was obtained from the area of peaks and the average chain length was also calculated. Eluents were prepared in distilled water with helium sparging: Eluent A was 500 mM sodium acetate in 150 mM NaoH, Elent B was 150 mM NaOH. Separation of linear components was achieved on a Dionex CarboPac^{TM} PA1 column with gradient elution 0 min, 40% eluent A; 5 min, 60% eluent A; 45 min, 80% eluent A) at a column temperature of 26°C and a flow rate of 1 ml/min.

3.2.5.2 Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)

Short range molecular order was determined using a Digilab FTS 7000 spectrometer (Digilab USA, Randolph, MA, USA) equipped with a thermoelectrically cooled deuterated triglycine sulphate (DIGS) detector using an attenuated total reflectance (ATR) accessory at a resolution of 4 cm^{-1} by 128 scans. Spectra were baseline-corrected and then

deconvoluted by drawing a straight line between 1200 and 800 cm^{-1} (using Win-IR Pro software, BioRad, Mississuaga, ON, Canada).

3.2.5.3 Wide angle X-ray diffraction (WAXS)

Relative crystallinity (RC) and polymorphic composition (proportion of A- and B-type unit cells) were calculated using a Rigaku Ultima IV X-ray diffractometer (Rigaku Americas, TX, USA) with operating conditions of target voltage 40 kV; current 44 mA; scanning range 3-35°; scan speed 1.00°/min; step time 0.95; divergence slit width 0.5°; scatter slit width 0.5°; sampling width 0.03° and receiving slit width 0.3mm. The moisture content of the starches was adjusted to ~21% by being kept in a desiccator over saturated K_2SO_4 solutions (a_w = 0.97) at room temperature for 14 days. RC (%) was calculated by a curve fitting procedure using IGOR pro 6.1 software (Wavemetrics Inc., Lake Oswego, OR, USA). A Gaussian function was used for curve fitting (Appendix 2). The polymorphic composition of the starches was determined as described by Ambigaipalan et al. (2011). Crystal size at the main reflections (5.5°, 17°, 23° 2θ) was determined using the Scherrer's equation (Langford & Wilson, 1978) as shown below:

$\boldsymbol{\tau} = \frac{k\lambda}{\beta cos\theta}$ (τ is the mean size of the ordered (crystalline) domains, K is a dimensionless shape factor, with a value close to unity, λ is the X-ray wavelength, β is the line broadening at half the maximum intensity, θ is the Bragg angle).

3.2.5.3.1 Determination of 'A' and 'B' polymorphic composition by X-ray diffraction

The proportion of 'A' and 'B' polymorphic compositions of the starches were determined by the method described by Zhou, Hoover and Liu (2004). The 'B' polymorph content was obtained by calculating the ratio of the area under the diffraction peak at 5.6° 2θ to the total crystalline area together with the calibration curve (Appendix II) obtained from mixtures of pure 'B' type (0-100% potato starch) and pure 'A' type (100-0% waxy corn starch).

3.2.6 Starch properties

3.2.6.1 Amylose leaching (AML)

Native starches (20 mg/db) in water (10 ml) were heated at 90°C in volume calibrated sealed tubes for 30 min. The tubes were then cooled to ambient temperature and centrifuged at 2000 x g for 10 min. The supernatant liquid (1 ml) was withdrawn and its amylose content determined as described by Hoover and Ratnayake (2001). AML was expressed as percentage of amylose leached per 100g of dry starch. Three replicate samples were used in this determination.

3.2.6.2 Differential scanning calorimetry (DSC)

Gelatinization parameters of starches were measured and recorded on Mettler-Toledo DSC equipped with a thermal analysis data station and data recording software (STAR@ SW 9.20). Water (11 μL) was added with a microsyringe to starch (3.0 mg db) in the DSC pans, which were then hermetically sealed, reweighed and allowed to stand overnight at

room temperature. The scanning temperature range and the heating rates were 30-110°C and 10°C/min, respectively. In all measurements, the thermogram was recorded with an empty aluminum pan as the reference. The transition temperatures reported are the onset (T_o), peak (T_p) and conclusion (T_c). The enthalpy of gelatinization (ΔH) was assessed by integrating the area between the thermogram and a base line under the peak and was expressed in terms of Joules per gram of dry starch. All DSC experiments were replicated thrice.

3.2.6.3 Rapid visco analyzer (RVA)

Rapid Visco Analyzer RVA-4 (Newport Scientific Pty. Ltd., Warriewood, NSW, Australia) was used to measure the pasting properties of pulse starches (10% db). The samples were held at 50°C for 1 min, heated at 6°C/min at 95°C, held at 95°C for 5 min, cooled subsequently at 6°C/min at 50°C, and held at 50°C for 2 min. The speed was maintained at 960 rpm for the first 10s, then to 160 rpm for the rest of the experiment.

3.2.6.4 Acid hydrolysis

Starches were hydrolyzed in triplicate with 2.2M HCl (1g, db, starch/40 ml) at 35°C in a water bath (New Brunswick Scientific, G76D, Edison, NJ, USA) for periods ranging from 1 to 25 days. Starch slurries were vortexed daily to re-suspend the deposited starch granules. Aliquots taken at specific time intervals were neutralized with 2.2M NaOH and centrifuged at 2000 x g for 10 min. The extent of hydrolysis was determined by expressing the solubilized carbohydrates (Jane, & Robyt, 1984) as a percentage of the initial starch using the method of Bruner (1964).

3.2.6.4.1 Determination of reducing value

2 ml of 3,5-dinitrosalicylic acid (DNS) was measured in a glass tube and placed in an ice-water bath for 5 min and an aliquot of sample (1 ml) was pipetted into 2 ml of the chilled 3.5-DNS. Then the reaction mixture was diluted to 4 ml with distilled water. The contents were mixed and placed in the ice-bath until thoroughly chilled and then was then subjected to heating for 5 min in a boiling water bath for color development. The contents were then returned to the ice-water bath before color measurement. After chilling, the final volume was brought to 8 ml with distilled water at room temperature. Contents were subjected to vortex mixing and the absorbance was measured at 540 nm. If the relative absorbance of the sample exceeded 1.5 at 540 nm, the spectrophotometer was set to 590 nm and then the relative absorbance was measured. The apparent sugar content of the sample was calculated by using the appropriate regression equation of the standard curves (Appendix III)

3.2.6.5 In vitro starch digestibility

In vitro starch digestibility was performed by the AACC International (2000) approved method 32-40. Pulse starches (100 mg) were incubated with 10 mg pancreatin and 12U amyloglucosidase in 4 ml of 0.1M sodium maleate buffer (pH 6.0) at 37°C with continuous shaking (200 strokes/min) for 0.5 – 16h and after incubation, 4 ml of 95% ethanol were added to terminate the reaction and the sample was centrifuged at 2000 x g for 10 min. Glucose content of the supernatant was calculated by a glucose oxidase-peroxidase assay kit (Megazyme International Ireland Ltd, Bray, Ireland). Based on the

rate of hydrolysis, starch was classified as rapidly digestible (digested within 0.5h), slowly digestible (digested between 0.5 and 16h) and resistant (undigested after 16h).

3.2.6.5.1 Determination of glucose content by Megazyme glucose method

GOPOD (Glucose oxidase/peroxidase) reagent buffer [1.0 M, pH 7.4, p-hydroxybenzoic acid and sodium azide (0.4% w/v)] was diluted with distilled water to a final volume of 1 l. GOPOD reagent enzyme (>12000 U) was dissolved in 20 ml of freshly prepared GOPOD buffer. 3 ml of GOPOD reagent was added to 0.1 ml of sample solution containing glucose and was incubated for 20 min at 50°C. The absorbance (ΔA) of the sample was then measured at 510 nm against reagent blank. The glucose content was calculated using the formula:

$$\text{Glucose } (\mu g/0.1 \text{ ml}) = \frac{\Delta A \text{ sample}}{\Delta A \text{ glucose standard } (100\ \mu g)} \text{ X } 100 \quad \text{.....(EQ 3.4)}$$

3.2.7 Retrogradation

3.2.7.1 Turbidity

A 2% aqueous suspension (200 mL) of starch was heated for 1 hr in a boiling water bath under continuous gentle stirring. It was then cooled for 20 min at 25°C. Triplicate samples were used and turbidity was determined by measuring transmittance at 640 nm against water blank with a Milton Roy UV–visible spectrophotometer (Spectronic 601, Milton Roy Company, Warminster, Pennsylvania, USA). The turbidity development was monitored by storing the remaining starch pastes for 1 day at 4°C followed by 2-7 days at 40°C.

3.2.8 Statistical analysis

Statistical analysis was performed among cultivars for each location and also between the means for each location. All determinations were replicated three times and the mean values and standard deviations were calculated. Analysis of variance (one-way ANOVA) was performed and the mean separations were carried out by Tukey's HSD test ($P<0.05$) using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

Chapter 4

Results and discussion

4.1 Chemical composition

Data on the yield and composition are presented in Table 1. The purity of the starches was judged on the basis of composition (low nitrogen) and microscopic observation (absence of any adhering protein). The yields (on a total seed basis) of pure starch (29.1 to 37.2%) were in the range reported (12.3 – 49.3%) for other pulse starches (Hoover et al., 2010). The nitrogen content was in the range 0.02 to 0.03%. The low nitrogen content indicated the absence of non-starch lipids (lipids associated with endosperm proteins). The apparent amylose content (AAM) of the cultivars (38.2 to 42.6%) were in the range reported (17.0 to 49.0%) for other pulse starches (Hoover et al., 2010). At both locations, differences in AAM among cultivars were marginal (Table 1). In all starches, the free and bound lipids ranged 0.05 – 0.13 and 0.97 – 1.29%, respectively. Starch damage was minimal (<1.0%) in all cultivars. The specific surface area (SSA) ranged from 0.31 to 0.38 m^2/g. There was no significant difference in SSA among cultivars at Rosthern. However, at Meathpark, ABAR exhibited a lower SSA (0.31 m^2/g) than the other cultivars (0.35 to 0.36 m^2/g). The arithmetic mean diameter (AMD) ranged from 26.4 to 30.5 μm. At both locations, ABAR exhibited a higher AMD (Table 1).

Table 4.1: Chemical composition (%) of field pea starches

Starch source and location	Yield (% initial material)	Nitrogen	Apparent amylose content[2]	Lipid		Arithmetic mean diameter (µm)	Specific surface area (m^2/g)
				Free[3]	Bound[4]		
Rosthern:							
CDC Golden	34.7	0.03±0.00[a]	40.6±0.0[a]	0.06±0.03[a]	1.11±0.00[a]	28.0±0.2[a]	0.38±0.01[a]
Abarth	36.9	0.03±0.00[ab]	38.4±0.3[b]	0.07±0.01[a]	1.10±0.40[a]	30.5±0.3[c]	0.36±0.04[a]
CDC Patrick	33.7	0.02±0.00[b]	40.1±0.2[a]	0.05±0.01[a]	1.29±0.12[a]	27.2±0.4[ab]	0.35±0.03[a]
CDC Amarillo	37.2	0.03±0.00[ab]	39.2±0.5[c]	0.12±0.01[a]	1.28±0.06[a]	27.1±0.1[ab]	0.36±0.01[a]
Mean		0.028[d]	39.58[d]	0.08[c]	1.20[c]	28.2[d]	0.36[d]
Meathpark:							
CDC Golden	35.2	0.03±0.00[e]	39.2±0.5[eh]	0.07±0.04[b]	1.27±0.01[b]	26.8±0.5[e]	0.35±0.01[ef]
Abarth	29.1	0.02±0.00[f]	38.2±0.7[ef]	0.07±0.00[b]	1.05±0.10[b]	30.0±0.0[f]	0.31±0.01[e]
CDC Patrick	35.2	0.03±0.00[e]	42.6±0.2[g]	0.13±0.04[b]	1.25±0.07[b]	26.4±0.0[e]	0.36±0.00[f]
CDC Amarillo	34	0.02±0.00[f]	39.8±0.3[h]	0.10±0.00[b]	0.97±0.20[b]	26.6±0.2[e]	0.35±0.02[f]
Mean		0.025[d]	39.95[d]	0.09[c]	1.14[c]	27.45[d]	0.34[d]

Statistical analysis was performed among cultivars for each location and also between the means for each location.

[1]All data represent the mean of triplicates. Values followed by the same superscript in each column for each starch source are not significantly different ($P<0.05$) by Tukey's HSD test.

[2]Determined by iodine binding without removal of free and bound lipids.

[3]Lipid extracted from the starch by chloroform-methanol (CM) 2:1 (v/v) at 25°C (mainly free lipids).

[4]Lipid extracted by hot 1-propanol-water (PW) 3:1 (v/v) from the residue left after CM extraction (mainly bound lipids).

4.2 Morphological characteristics

Scanning electron microscopy (Fig. 1, supplementary) showed that granules from all four cultivars had irregular shapes, which varied from elliptical to round. The surfaces of the above starches appeared to be smooth. However, bright field microscopy (Figs 2a–f, supplementary) showed the presence of cracks on many granules of all four cultivars. The nature and extent of cracking varied among granules. Cracks have also been reported in other pulse starches (Ambigaipalan et al., 2011). Cracking has been shown to reflect low granule integrity (due to weak interaction between radially arranged AP chains) resulting in an increase in strain as the granule grows, and/or to sub-optimal packing of starch chains within the granule (Glaring, Koch, & Blennow, 2006). The birefringence patterns (Fig 3 a-h, supplementary) viewed under polarized light reflects the radial arrangement of AP crystallites within the granule at right angles to the surface with their single reducing end group towards the hilum. Birefringence implies that there is a high degree of molecular orientation without reference to the three dimensional crystalline order. At both locations, CDCA (Figs 3f, h) exhibited strong birefringence in many of its granules (arrow 1), well defined quadrants (arrow 2) in some granules and disorganized quadrants (arrow 3) in others. However, in CDCG (Figs 3 a, c), ABAR (Figs 3b, d) and CDCP (Figs 3e, g) birefringence intensities were weaker (arrow 4) and many granules exhibited disorganized quadrants (arrow 5) with large voids at the granule centre (arrow 6). The intensity of the birefringence pattern has been shown to be influenced by AP content, irregularly shaped granules, disrupted crystallites and radial orientation of AP chains (Ambigaipalan et al., 2011). In this study, differences in AP content (Table 1) and granule

morphology (Figs 1 & 2 supplementary) were not significant. Furthermore, disrupted crystallites cannot be a factor influencing birefringence, since relative crystallinity (Table 3) differences among the cultivars (CDCG > ABAR > CDCP ~CDCA) did not correlate with birefringence intensities (CDCA > CDCG~CDCP~ABAR). This suggests a greater degree of order in the molecular orientation of AP crystallites in CDCA.

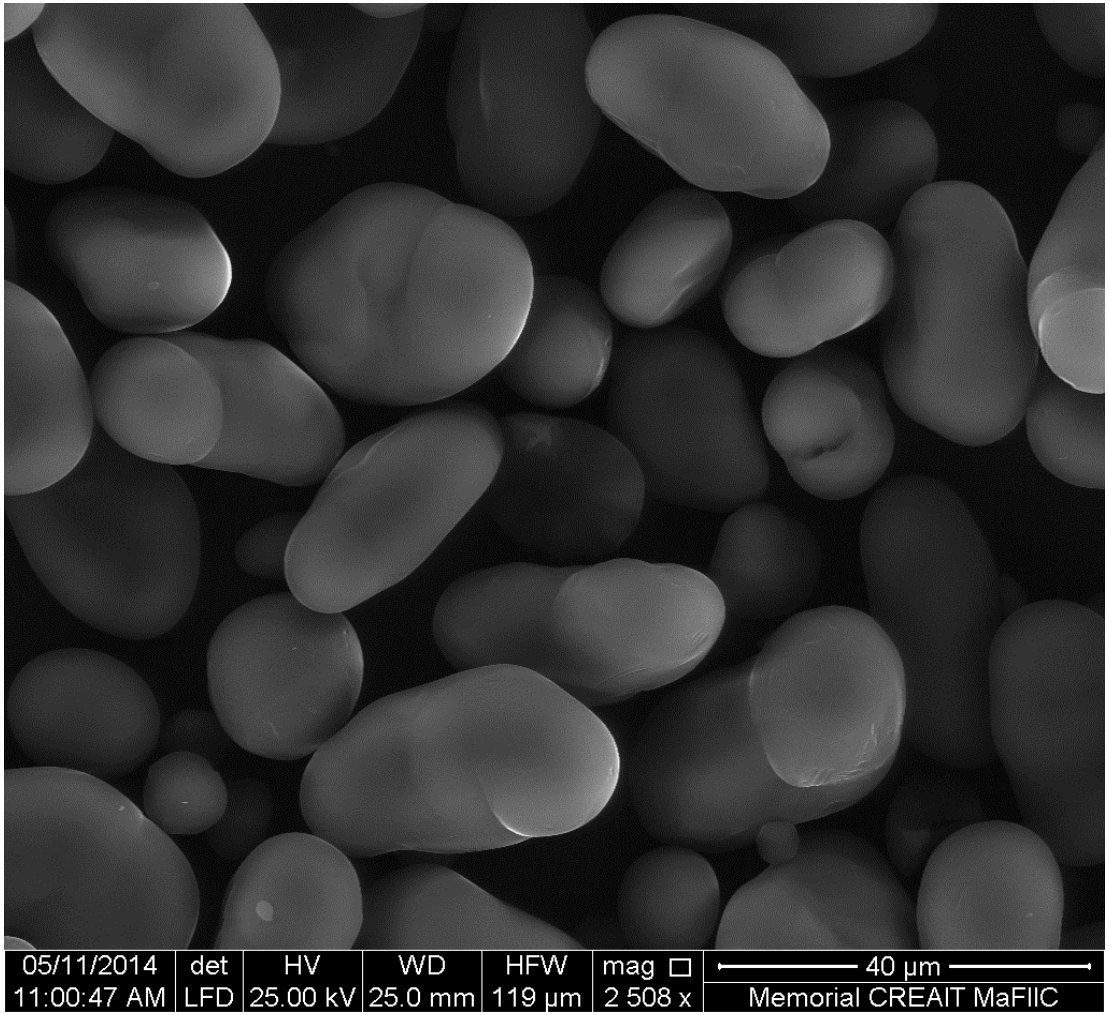

Figure 4.1: Scanning electron micrograph image of CDC Golden Rosthern

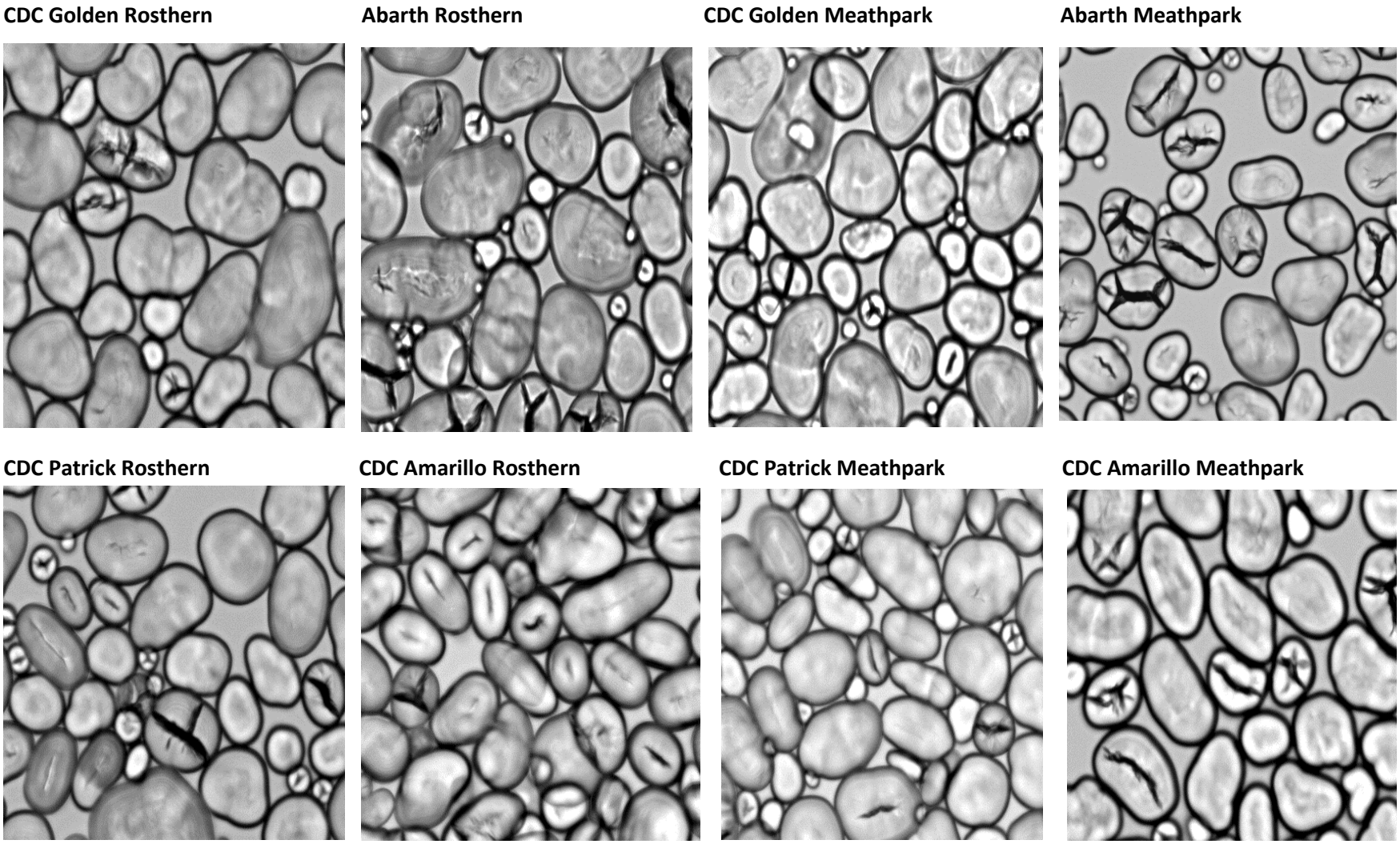

Figure 4.2: Bright field microscopy images of field pea starch

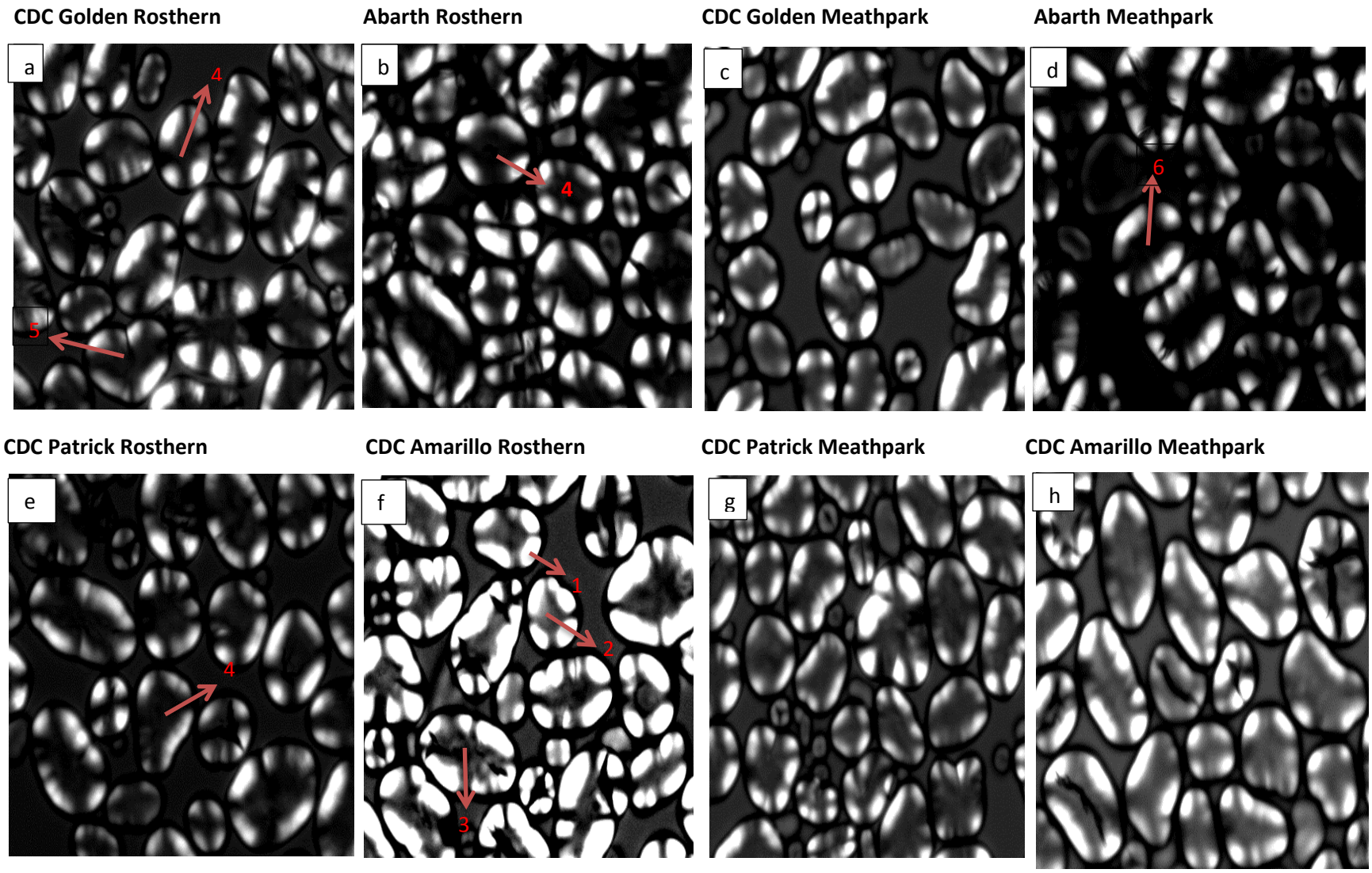

Figure 4.3: Polarized light microscopy images of field pea starches. Arrows 1 – 3 indicate quadrants, voids and shape of the quadrants

4.3 Amylopectin chain length distribution (APCLD)

The normalized branched APCLD of the four cultivars are presented in Table 2. At both locations, CDCG and ABAR differed significantly ($P<0.05$) from CDCP and CDCA with respect to a higher proportion of short chains [SC (DP 6-12)], lower proportion of long chains [LC (DP 25-36)], lower average chain length ($\overline{CL}$), and higher SC/LC ratio (reflects high degree of AP branching). However, there was no significant ($P<0.05$) difference among the cultivars with respect to maximum detectable DP and in the proportion of chains with DP<6, DP (13-24) and DP>37. The APCLD of these starches were in the range reported for other pulse starches (Hoover et al., 2010). The SC/LC ratio (CDCG~CDCA > CDCP~CDCA) has been shown to reflect variable activity, due to gene mutations, of the enzymes involved in starch synthesis (Zhang, Ao, & Hamaker, 2008).

Table 4.2: Amylopectin chain length distribution of field pea starches determined by high performance anion exchange chromatography with pulsed amperometric detection[1]

Starch source	Degree of polymerization[2] (dP)%							
	<6	6-12 (A chains)	13-24 (B1 chains)	25-36 (B2 chains)	>37 (B3 chains)	$\overline{CL}$[3]	SC/LC[4]	Maximum detectable dP
Rosthern								
CDC Golden	0.4±0.3^{a}	25.4±0.0^{a}	48.9±0.0^{a}	14.1±0.1^{a}	10.9±0.0^{a}	16.3±0.5^{a}	0.34	59
Abarth	0.4±0.3^{a}	24.7±0.0^{b}	49.1±0.0^{a}	14.2±0.0^{a}	11.5±0.2^{a}	16.0±0.2^{a}	0.33	65
CDC Patrick	0.5±0.2^{a}	19.8±0.0^{c}	52.9±1.2^{a}	16.4±0.0^{b}	11.4±0.4^{a}	16.8±0.2^{a}	0.25	64
CDC Amarillo	0.3±0.1^{a}	18.0±0.2^{d}	52.2±0.0^{a}	17.3±0.0^{c}	11.3±1.0^{a}	17.1±0.1^{a}	0.23	62
Mean	0.4^{e}	22.0^{e}	51.1^{e}	15.5^{e}	11.6^{e}	16.6^{e}		
Meathpark								
CDC Golden	0.5±0.3^{f}	25.0±0.7^{f}	49.2±1.2^{fg}	14.3±0.1^{f}	10.9±0.3^{f}	15.8±0.2^{f}	0.34	60
Abarth	0.6±0.3^{f}	24.8±0.8^{f}	48.9±0.6^{f}	14.0±0.4^{f}	11.7±0.0^{f}	15.8±0.3^{f}	0.34	64
CDC Patrick	0.6±0.2^{f}	19.1±0.1^{g}	51.9±0.0^{gh}	16.0±0.3^{g}	13.5±1.0^{f}	17.0±0.0^{g}	0.24	64
CDC Amarillo	0.4±0.1^{f}	18.9±0.2^{g}	53.3±0.0^{h}	16.7±0.2^{g}	11.9±1.7^{f}	16.9±0.2^{g}	0.24	62
Mean	0.5^{e}	22.0^{e}	50.8^{e}	15.3^{e}	12.0^{e}	16.4^{e}		

Statistical analysis was performed among cultivars of each starch source and also among the means of each starch source.

[1]All data represent the mean of triplicates. Values followed by the same superscript in each column for each starch source are not significantly different (P > 0.05) by Tukey's HSD test.

[2]DP*n*: indicates degree of polymerization. Total relative area was used to calculate the percent distribution.

[3]$\overline{CL}$ represent chain length.

[4]SC/LC represents the ratio of short chains (DP<6 to 12) to long chains (DP 13 to >37).

4.4 Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)

In ATR-FTIR spectroscopy, the vibrational modes influencing absorption bands at 1105, 1125 and 1155 cm^{-1} (C-OH, C-C and C-O stretching), 928, 995, 1048, 1080 cm^{-1} (C-OH bending and CH_2 vibrational modes), and 860 cm^{-1} (C-O-C symmetrical stretching and C-H deformation) reflect conformational and crystalline order in starch (van Soest, Tournois, de Wit, & Vliegenthart, 1995). The ATR-FTIR spectrum is sensitive to short range order, defined as the double helical order, as opposed to long range order related to the packing of double helices (detected by X-ray diffraction). The IR beam penetrates only to a depth of 2 micrometers of the sample (Sevenou, Hill, Farhat, & Mitchell, 2002). Hence, the IR spectra reflect short range order at or near the granule surface. The absorbance bands at 1022/1048cm^{-1} are characteristic of amorphous and crystalline structures in starch, respectively (Van Soest et al., 1995). Thus, the ratio of 1048/1022 cm^{-1} has been used to express the amount of ordered crystalline domains to amorphous domains in starches (Capron, Robert, Colonna, Brogly, & Planchot, 2007). As shown in Table 3, at both locations, the amount of ordered crystalline domains (at or near the granule surface) among the cultivars did not follow the same trend. For instance, at Rosthern and Meathpark, the 1048/1022 cm^{-1} ratio followed the order: CDCG~ABAR > CDCP ~CDCA and ABAR~CDCG~CDCA > CDCP, respectively. The FTIR ratio was within the range 0.778 to 1.16 reported for other pulse starches (Maaran, Hoover, Vamadevan, Waduge, & Liu, 2016; Ambigaipalan et al., 2011).

4.5 Wide angle X-ray diffraction (WAXS)

The WAXS patterns of starch granules result from parallel packing of left handed co-axial double helices arranged in extended regular arrays. Granule crystallinity is believed to result from clustered AP chains of DP 13 - 15. Relative crystallinity (RC) and B-polymorphic content are presented in Table 3. All starches exhibited the "C" type X-ray pattern characteristic of pulse starches. The "C" type pattern has been shown (Hoover & Ratnayake, 2002) to be a mixture of "A" and "B" unit cells in varying proportions. The X-ray pattern was characterized by peaks at 5.6, 10.4, 15.0, 17.3, 19.9 and 23.3° 2θ. The B-polymorphic content ranged from 45.1 to 55.5% (ABAR > CDCA > CDCP > CDCG), and 41.0 to 55.4% (CDCG > CDCP > ABAR > CDCA) at Rosthern and Meathpark, respectively. At both locations, the B-polymorphic content not only varied significantly ($P<0.05$) among the cultivars, but was also significantly ($P<0.05$) different for each cultivar at the two locations (Table 3). The B-polymorphic content of pulse starches has been reported to be in the range of 22.1 to 48.0% (Hoover et al., 2010). The RC ranged from 33.1 to 36.8 (CDCG > ABAR > CDCA > CDCP) and 32.3 to 35.1 (CDCG ~ABAR > CDCP > CDCA) at Rosthern and Meathpark, respectively. In all cultivars, the crystallite size at 5.6, 17.3, 23.3° 2θ was 120, 59 and 45Å, respectively. RC differences among pulse starches could be influenced by: 1) crystallite size, 2) orientation of crystallites to the X-ray beam, 3) degree of packing of double helices within the crystalline lamella, 4) presence of a high proportion of short AP chains (DP<6), 5) extent of AP branching, and 6) moisture content. As shown in Table 2, among cultivars, the proportion of short chains (DP 6 -12) and the degree of AP branching was higher in

CDCG and ABAR. Therefore, theoretically, the RC of CDCG and ABAR should have been lower than those of CDCP and CDCA. This is indicative that the higher RC of CDC Golden and Abarth are mainly due to their crystallites being better oriented to diffract X-rays. This seems plausible, since AP content (Table 1), crystallite size and starch moisture content did not differ significantly ($P<0.05$) among cultivars.

Table 4.3: FTIR intensity ratio (1048/1016 cm^{-1}), relative crystallinity and B-polymorphic content of field pea starches[1]

Starch source and location	FTIR ratio 1048/1016 cm^{-1}	Relative crystallinity[2](%)	B polymorphic composition[3] (%)
Rosthern:			
CDC Golden	0.937±0.002^{a}	36.8±0.0^{a}	45.1±0.1^{a}
Abarth	0.934±0.003^{a}	34.1±0.1^{b}	55.5±0.1^{b}
CDC Patrick	0.924±0.001^{b}	33.1±0.0^{c}	46.1±0.0^{c}
CDC Amarillo	0.922±0.002^{b}	33.6±0.0^{d}	51.6±0.0^{d}
Mean	0.929^{f}	34.4^{i}	49.58^{i}
Meathpark:			
CDC Golden	0.938±0.000^{cd}	35.1±0.1^{e}	55.4±0.1^{e}
Abarth	0.946±0.005^{c}	35.1±0.0^{e}	50.9±0.1^{f}
CDC Patrick	0.913±0.001^{e}	34.0±0.0^{f}	53.4±0.0^{g}
CDC Amarillo	0.935±0.002^{d}	32.3±0.0^{g}	41.0±0.0^{h}
Mean	0.933^{f}	34.13^{i}	50.18^{i}

[1]All data represent the mean of triplicates. Values followed by the same letter, in the same column are not significantly different ($P<0.05$) by Tukey's HSD test. The moisture content of the starches were in the range of 16 - 17% (w/w)

[2].% crystallinity = $\Sigma|I_s-I_a|/|I_c-I_a|X100$, where I_s-I_a = difference between the sample and amorphous intensities and I_c-I_a = difference between the 100% crystalline (quartz) and amorphous intensities.

[3] Proportion of 'B' polymorph α [area of the peak at 5.54 (2θ)/total peak area].

4.6 Differential scanning calorimetry (DSC)

Gelatinization transition temperatures (T_o (onset), T_p (midpoint), and T_c (conclusion)), and enthalpies of gelatinization (ΔH) of the four pea cultivars are presented in Table 4. At both locations, CDCG and ABAR exhibited higher T_o, T_p, T_c, and a wider T_c - T_o, than those of CDCP and CDCA. However, there was no significant difference ($P<0.05$) in ΔH among cultivars. Gelatinization parameters have been shown to be influenced by the molecular architecture of the crystalline region which is related to the distribution of AP short chains (DP 6-11) and not by the proportion of crystalline region which corresponds to the AM/AP ratio (Noda, Takahata, Sato, Ikoma & Mochida, 1996). The above authors have shown that a low T_o, T_p, T_c and ΔH reflect the presence of abundant short AP (DP 6 – 13) chains. Shi and Seib (1992) have also shown by studies on ae, wx, ae, duwx maize starches, that wx starch having the lowest proportion of short chains (DP 6-13) exhibited lower T_o, T_p, T_c and ΔH values. Perez and Bertoft (2010) and Biliaderis, Grant and Vose (1981) have shown that starches with a high degree of AP branching exhibit low To, T_p, and T_c. However, in this study, the higher T_o, T_p, T_c were exhibited by CDCG and ABAR, where AP branching was more extensive and the proportion of short (DP 6 -12) and long (DP 25 – 36) chains were higher and lower, respectively, than those of CDCP and CDCA (Table 2). This suggests that AM may be co-crystallized with AP in CDCG and ABAR. This seems plausible, due to their higher proportion of short A chains (DP 6-12) and higher AP branching density (facilitates interactions with AM chains). Studies on waxy starches (Bertoft, Annor, Shen, Rumpagaporn, Seetharaman, & Hamaker, 2016) have shown that crystalline A chains (DP>9) potentially participate in the formation of double

helices. Thus, the higher T_o, T_p, and T_c of CDCG and ABAR is a reflection of the additional thermal energy required to disrupt AM – AP crystallites. The wider T_c-T_o exhibited by the above starches could be attributed to melting of both heterogenous (AP – AM) and homogenous (AP –AP) crystallites. This seems plausible, since there was no significant differences ($P<0.05$) among cultivars with respect to AP content (Table 1) or crystal size (Section 3.4), and RC was mainly indicative of crystallite orientation rather than to differences in crystalline stability (Section 3.4). As shown in Table 4, there was no significant difference ($P<0.05$) in ΔH among the starches. Theoretically, ΔH should have been lower in CDCG and ABAR due to: 1) higher proportion of DP 6-12 chains and 2) higher degree of AP branching (prevents close alignment of double helices within the crystalline lamella). The data suggests that double helices resulting from AM-AP co-crystallization may have negated the impact of factors 1 and 2 on ΔH.

Table 4.4: Gelatinization parameters of field pea starches[1]

Starch source and location	Gelatinization transition parameters (°C)[2]				
	To[3]	Tp[3]	Tc[3]	(Tc-To)[4]	ΔH/(AP)[5] J/g
Rosthern:					
CDC Golden	62.2±0.1^{ab}	66.6±0.1^{ab}	74.7±0.4^{a}	12.5±0.5^{a}	5.8±0.1^{a}
Abarth	62.6±0.1^{a}	67.0±0.0^{a}	75.4±0.1^{a}	12.8±0.0^{a}	6.2±0.0^{b}
CDC Patrick	61.2±0.1^{c}	65.3±0.1^{c}	72.4±0.3^{b}	11.2±0.2^{b}	6.2±0.0^{b}
CDC Amarillo	61.8±0.2^{b}	66.0±0.3^{b}	72.8±0.4^{b}	11.0±0.2^{b}	6.0±0.1^{ab}
Mean	62.0^{d}	66.2^{d}	73.8^{d}	11.9^{d}	6.1^{d}
Meathpark:					
CDC Golden	61.3±0.1^{ef}	65.6±0.0^{e}	74.8±0.1^{e}	13.5±0.1^{e}	5.8±0.3^{e}
Abarth	61.5±0.3^{e}	65.8±0.2^{e}	74.9±0.0^{e}	13.3±0.3^{e}	6.3±0.1^{e}
CDC Patrick	60.6±0.2^{gh}	64.2±0.3^{f}	71.7±0.1^{f}	11.1±0.3^{f}	6.2±0.1^{e}
CDC Amarillo	60.8±0.0^{fh}	64.7±0.1^{f}	72.3±0.1^{g}	11.5±0.1^{f}	6.4±0.2^{e}
Mean	61.1^{d}	65.1^{d}	73.4^{d}	12.3^{d}	6.2^{d}

Statistical analysis was performed among cultivars for each location and also between the means for each location.

[1]All data represent the mean of triplicates. Values followed by the same superscript in each column for each starch source are not significantly different ($P<0.05$) by Tukey's HSD test.

[2]Starch: water ratio = 1:3 (w/w dry basis).

[3]To, Tp, and Tc indicates the onset, peak, and end temperature, respectively.

[4](Tc-To) indicates the gelatinization temperature range.

[5]Gelatinization enthalpy expressed on the basis of amylopectin content.

4.7 Amylose leaching (AML)

The extent of AML at 90°C is presented in Table 5. At both locations, AML was less pronounced in CDCG and ABAR than in CDCP and CDCA. AML has been shown to be influenced by: A) bound lipid content, B) amylose content, and C) extent of interaction between AM-AM and AM-AP chains (Hoover & Ratnayake, 2002). In this study, there was no significant difference in A or B among the starches (Table 1). Therefore, the lower extent of AML observed with CDCG and ABAR lends credence to our postulate that AM is co-crystallized with AP in the above starches.

4.8 Pasting properties

Pasting is the phenomenon following gelatinization in the dissolution of starch. The granules become increasingly susceptible to shear disintegration as they swell and release soluble material as they disintegrate. The paste that is obtained on gelatinization is a viscous mass consisting of a continuous phase of solubilized AM and/or AP, and a discontinuous paste of granule remnants. The peak viscosity and shear stability have been shown to be influenced by AM content, proportion of AP chain lengths of DP 13-24 and DP>37 (Han & Hamaker, 2001). Han and Hamaker (2001) have shown by studies on rice starches of nearly similar content, but differing significantly in the proportion of long AP chains, that rice starches containing the highest proportion of long AP chains showed almost no viscosity breakdown. This was attributed to the entanglement between long AP chains (decreases the extent of dispersion) and to an increase in the gyration radius (volume occupied by AP in a solution) of AP molecules. Final viscosity has been shown to be influenced by: 1) extent of AML (during the heating period), 2) network formation by leached AM chains, 3) presence of intact rigid swollen granules embedded in the AM network (Maaran, Hoover, Donner, & Liu, 2014). The pasting properties of the starches are presented in Table 5. At both locations, the difference in breakdown viscosity (BV) and final viscosity (FV) among cultivars, followed the same order: CDCG > ABAR > CDCP ~CDCA, and CDCP > CDCA > CDCG > ABAR, respectively. However, the same trend was not seen with respect to pasting temperature (PT), peak viscosity (PV), and setback (SB) at the two locations. For instance, at Rosthern, PT, PV and SB followed the order: CDCG ~ABAR > CDCA ~CDCP, CDCG > CDCP > ABAR >CDCA and CDCG

> CDCA > CDCP > ABAR, respectively. Whereas, at Meathpark, the corresponding orders were CDCG > ABAR > CDCA~CDCP, CDCP > CDCG > CDCA > ABAR and CDCP > CDCA~CDCG > ABAR, respectively. The greater thermal stability exhibited by CDCP and CDCA (reflected in their lower BV) could be attributed to their higher proportion of long AP chains (DP 13 to 36) and smaller proportion of short (DP<12) AP chains (Table 2). The higher FV exhibited by both CDCP and CDCA, is indicative that their lower extent of granule breakdown during the holding period may have resulted in a large proportion of intact granules being embedded within the AM network. The presence of these embedded intact granules may have imparted resistance to the stirring action of the RVA paddles during the cooling cycle, resulting in a higher FV for the above starches. In many starches, the SBV order normally mirrors the FV order. However, this was not the case with CDCP (at Rosthern) and CDCG (at Rosthern and Meathpark).

Table 4.5: Amylose leaching and pasting properties of field pea starches[1]

Starch source and location	Amylose leaching at 90°C (%)	Pasting temperature(°C)	Peak viscosity(cP)[2]	Breakdown viscosity(cP)[2]	Final viscosity(cP)[2]	Setback viscosity(cP)[2]
Rosthern:						
CDC Golden	11.8±0.0	72.7±0.0^{a}	4428±3^{a}	2363±67^{a}	4730±61^{a}	2665±9^{a}
Abarth	9.8±0.0	72.3±0.5^{a}	3332±14^{b}	1510±20^{b}	3958±37^{b}	2136±4^{b}
CDC Patrick	14.0±0.0	69.8±0.6^{b}	3839±32^{c}	1217±4^{c}	5193±54^{c}	2571±18^{c}
CDC Amarillo	13.6±0.0	70.3±0.0^{b}	3254±41^{b}	885±31^{d}	4946±47^{d}	2577±37^{c}
Mean	12.3	71.28^{h}	3713.25^{h}	1493.75^{h}	4706.75^{h}	2487.25^{h}
Meathpark:						
CDC Golden	7.3±0.0	72.5±0.3^{e}	4349±11^{e}	2397±45^{e}	4442±82^{e}	2491±26^{e}
Abarth	11.4±0.0	71.9±0.0^{f}	3284±12^{f}	1772±37^{f}	3437±4^{f}	1926±53^{f}
CDC Patrick	13.7±0.0	69.8±0.6^{g}	4663±24^{g}	1624±3^{g}	6026±65^{g}	2987±38^{g}
CDC Amarillo	14.2±0.0	70.3±0.1^{g}	3581±16^{h}	981±23^{h}	5048±40^{h}	2448±1^{e}
Mean	11.7	71.13^{h}	3969.25^{h}	1693.5^{h}	4738.25^{h}	2463^{h}

Statistical analysis was performed among cultivars for each location and also between the means for each location.

[1] All data represent the mean of triplicates. Values followed by the same superscript in each column for each starch source are not significantly different ($P<0.05$) by Tukey's HSD test.

[2] cP refers to centipoise.

4.9 Acid hydrolysis

Acid hydrolysis of the starches by 2.2M HCl during a 25 day period is presented in Table 6. At both locations, differences in the extent of hydrolysis among the starches during the first eight days of hydrolysis were marginal. However, on day 12, CDCP and CDCA were hydrolyzed to a lesser extent (34.2 to 38.0%) than CDCG and ABAR (44.6 to 47.1%). Furthermore, the extent of increase in hydrolysis between days 8 and 12, was much higher in CDCG and ABAR (29.6 to 44.7%) than in CDCP and CDCA (32.1 to 34.2%). On day 25, CDCG and ABAR were hydrolyzed to a greater extent than CDCP and CDCA. These differences were more marked at Meathpark. It has generally been accepted that heterogeneous acid hydrolysis preferably attacks the bulk and intercrystalline amorphous regions of the granule. In contrast, crystalline regions are less accessible to attack by hydrolytic protons and are attacked only after a period of 10 to 12 days (Hoover, 2000). To account for the slower hydrolysis of the crystalline regions, two hypotheses have been proposed (Hoover, 2000). First, the dense packing of starch chains within the starch crystallites hinders the penetration of H_3O^+. Second, acid hydrolysis requires a ring distortion of the $^4C_1$ conformation of the glucose units to the half chain conformation. In the half chair conformation, the glycosidic linkages become more accessible to H_3O^+. Immobilization of the sugar conformation due to dense packing of AP double helices in the crystalline domains would render the above transition difficult. The slower increase in hydrolysis between days 8 and 12 seen with CDCP and CDCA in Table 6 is indicative that glycosidic linkages present in crystallites of the above starches are less accessible than those present in CDCG and ABAR to attack by H_3O^+. As shown

in Table 2, the AP chains of both CDCP and CDCA were composed of fewer proportions of short chains (DP 6-12), larger proportion of longer chains (DP 25-36) and higher average chain length ($\overline{CL}$) than those of CDCG and ABAR. Therefore, it is likely, that in CDCP and CDCA, their longer DP 25-36 chains (represent B2 chains which span two amylopectin clusters) may have been entangled with other AP molecules, rendering the $^4C_1$ to half chair conformation difficult. This would then restrict the ability of H_3O^+ to access the glycosidic linkages. In summary, the differences in hydrolysis among the starches between days 8 and 12 are probably influenced by differences in their proportion of short chains, long chains and average chain length (Table 2).

Table 4.6: Acid hydrolysis (%) of field pea starches[1]

Starch source and location	Number of days						
	1	2	5	8	12	20	25
Rosthern:							
CDC Golden	2.3±0.0^{a}	6.1±0.2^{a}	20.3±0.0^{ab}	29.7±0.5^{a}	44.6±0.1^{a}	60.2±0.3^{a}	69.3±0.6^{a}
Abarth	2.3±0.0^{a}	6.3±0.0^{a}	21.7±0.1^{ac}	29.6±0.2^{a}	44.7±0.1^{a}	60.7±0.7^{a}	71.9±0.0^{b}
CDC Patrick	1.7±0.0^{b}	5.7±0.0^{b}	19.8±0.7^{b}	32.1±0.4^{b}	34.2±0.5^{b}	59.9±0.3^{a}	66.8±0.4^{c}
CDC Amarillo	2.0±0.0^{c}	6.3±0.0^{a}	22.0±0.3^{c}	32.1±0.1^{c}	38.0±0.7^{c}	61.7±1.2^{a}	68.7±0.2^{a}
Meathpark:							
CDC Golden	2.3±0.1^{d}	6.4±0.0^{d}	21.8±0.1^{d}	30.2±0.1^{d}	46.8±0.2^{d}	63.2±0.1^{d}	74.2±0.3^{d}
Abarth	2.6±0.0^{e}	6.6±0.0^{e}	22.3±0.1^{e}	31.9±0.2^{d}	47.1±0.0^{d}	64.6±0.1^{e}	75.9±0.3^{e}
CDC Patrick	1.8±0.0^{f}	6.1±0.0^{f}	19.7±0.1^{f}	31.9±0.9^{e}	37.4±1.2^{e}	61.0±0.4^{f}	67.8±0.6^{f}
CDC Amarillo	2.1±0.0^{g}	6.1±0.0^{f}	19.1±0.0^{g}	31.4±0.1^{f}	36.2±0.2^{e}	60.6±0.3^{f}	64.3±0.1^{g}

[1]All data represent the mean of triplicates. The values followed by the same superscript are not significantly different ($P<0.05$) by Tukey's HSD test.

4.10 Starch digestibility

Rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) determined by in vitro digestibility of the field pea starches by a mixture of pancreatin and amyloglucosidase are presented in Table 7 (supplementary). The above nutritional classification reflects both the kinetic component and the completeness of digestibility. RDS reflects starch that is rapidly and completely digested in the small intestine. SDS reflects starch that is more slowly digested in the small intestine and RS reflects the sum of starch, and the product of starch degradation not absorbed in the small intestine, but is fermented in the large intestine. (Jenkins et al, 1983). As shown in Table 7 (supplementary), at both locations, CDCP and CDCA exhibited RDS levels (10.4 to 10.9%) that were higher than those of CDCG and ABAR (8.6 to 9.5%). These levels were in the range (8.1 to 23.8%) reported for other pulse starches (Chung, Liu, Hoover, 2009; Miao, Zhang, Jiang, 2009; Sandhu & Lim, 2008). Variations in SDS levels among cultivars ranged from 44.9 – 48.8% (Rosthern) and 43.7 – 48.8% (Meathpark). These levels were higher than those reported (29.7 to 45.7%) for other pulse starches (Hoover et al., 2010). The RS levels at Rosthern (28.9 – 34.9%) and Meathpark (29.7 – 38.7%) followed the order of CDCG > ABAR > CDCP ~CDCA, and CDCG > ABAR > CDCA > CDCP, respectively. The range in RS levels for the above cultivars was lower than the range (40.5 to 65.2%) reported for other pulse starches (Hoover et al., 2010). The rate and extent of enzyme hydrolysis has been shown (Hoover & Zhou, 2003; Zhang, Venkatachalam & Hamaker, 2006) to be influenced by: 1) granule surface area, 2) pores on the granule surface, 3) relative crystallinity, 4) crystal size, 5) polymorphic content, 6)

AM content, 7) proportion of short AP chains, 8) AP branching density, and 9) molecular order at the granule surface, 10) degree and extent of retrogradation between hydrolyzed starch chains. In this study, differences in RDS levels among cultivars are mainly influenced by the interplay among differences in: a) molecular order (ABAR > CDCG > CDCA > CDCP), b) extent of AP branching (CDCG ~ ABAR > CDCP ~ CDCA) and c) proportion of AP short chains (CDCG ~ ABAR > CDCP ~ CDCA). A high degree of molecular order would restrict diffusion of amylolytic enzymes into the granule interior. Whereas, extensive AP branching and a high proportion of short AP (DP 6 -12) chains would restrict the ability of amylolytic enzymes to access the glycosidic linkages. This seems plausible, since differences in RDS levels among cultivars cannot be explained in terms of factors 1 to 6 due to the following reasons: 1) absence of pores on the granule surfaces (Fig 1, supplementary), 2) marginal differences in AM content (Tables 1, & 3) similar crystallite size Section 3.4), 4) RC differences did not reflect AP double helical packing density within the crystalline lamella, and 5) Gérard, Colonna, Buléon, Planchot (2001) have shown that regardless of AM content or RC, starches with a higher proportion of B-type crystallites are more resistant to amylolysis than A or C-type (contain A and B polymorphs in varying proportion) crystallites. However, in this study, variation in B-polymorphic content (Table 3) among cultivars cannot be considered as a factor influencing RDS levels, since at both locations, there was no correlation between RDS levels and B-polymorphic content. For instance, at Meathpark, CDCP and CDCA with B-polymorphic contents of 53.4 and 41.0%, respectively, exhibited similar RDS levels. Whereas, at Rosthern, CDCA with a higher B-polymorphic content (51.0%) than CDCG (41.0%) exhibited a higher RDS level (Table 7, supplementary).Variation in RS

content among cultivars (CDCG˜ABAR > CDCP˜CDCA) reflect the interplay among the following factors: A) rate and extent of aggregation of double helical segments formed by retrogradation of hydrolyzed amylose chains with a DP in the range 30 to 70 (Leloup, Colonna, & Ring, 1991), B) state of organization of AM chains within native granules (free or co-crystallized with other AM or AP chains) and C) AP branching density. It is plausible, that the higher resistance exhibited by CDCG and ABAR towards amylolysis may be influenced by their higher AP branching density (slows digestion due to the slower kinetics associated with digestion of α (1→6) linkages (Zhang & Hamaker, 2013) and AM-AP co-crystallization (sterically hinders the conformational transition (chair to half-chair) required for hydrolysis of the glucosidic linkages of the D-glucopyranosyl units).

Table 4.7: Nutritional fractions of field pea starches determined by *in vitro* hydrolysis[1]

Starch source and location	RDS[2]	SDS[2]	RS[2]
Rosthern:			
CDC Golden	9.2±0.3[a]	45.9±0.6[a]	34.9±0.3[a]
Abarth	8.6±0.3[a]	47.9±0.4[b]	31.1±0.1[b]
CDC Patrick	10.5±0.0[b]	44.9±0.4[a]	28.9±0.4[c]
CDC Amarillo	10.9±0.2[b]	48.8±0.3[b]	29.9±0.4[bc]
Mean	9.8[e]	46.9[e]	31.2[e]
Meathpark:			
CDC Golden	8.6±0.2[f]	43.7±0.3[f]	38.7±0.5[f]
Abarth	9.5±0.1[g]	48.8±0.3[g]	35.6±0.1[g]
CDC Patrick	10.8±0.3[h]	47.8±0.2[g]	29.7±0.5[h]
CDC Amarillo	10.4±0.0[h]	48.2±0.5[g]	33.8±0.5[i]
Mean	9.8[e]	47.1[e]	34.5[e]

Statistical analysis was performed among cultivars for each location and also between the means for each location.

[1] All data represent the mean of triplicates. Values followed by the same superscript in each column for each starch source are not significantly different ($P<0.05$) by Tukey's HSD test.

[2] RDS: rapidly digestible starch; SDS: slowly digestible starch; RS: resistant starch

4.11 Turbidity

Turbidity effects have their origin in refractive index fluctuations over a distance scale comparable to the wavelength of observation (Gidley & Bulpin, 1987). In a polymer-solvent system this is caused by density fluctuations over the same distance scale, and is most likely due to extensive polymer-polymer aggregation. Turbidity measurements have been used extensively to determine the rate and extent of starch retrogradation (Ambigaipalan, Hoover, Donner, & Liu, 2013; Karim, Norziah, & Seow, 2000. The first stage of retrogradation has been shown to involve mainly interaction between amylose (AM) chains leading to crystallization, which is completed within the first few hours of storage, whereas, the second stage that occurs over longer periods involves primarily interaction between AP chains leading to crystallization. As illustrated in figure 4 (supplementary), at both locations, the %T on day 0 followed the order: CDCG>ABAR>CDCP~CDCA. %T decreased rapidly in all starches (ABAR~ CDCG>CDCP>CDCA) during the first 24h of storage. Thereafter, %T decreased gradually to the same extent (Fig 4). The low %T exhibited by both CDCP and CDCA at day 0 could be attributed to their higher extent of AML (Table 5) and to the presence of a higher proportion of intact swollen granules in the gelatinized paste (Section 3.8). The rapid decrease in %T in all starches during the first 24h of storage reflects the interplay between the extent of AML and the rate and extent of interaction between leached amylose chains (influenced by the viscosity of the gelatinized paste). The absence of any significant difference in %T beyond 24h of storage is indicative that the rate of

interactions between AM-AM, AM-AP and AP-AP chains may have been too slow to be measurable by light transmission.

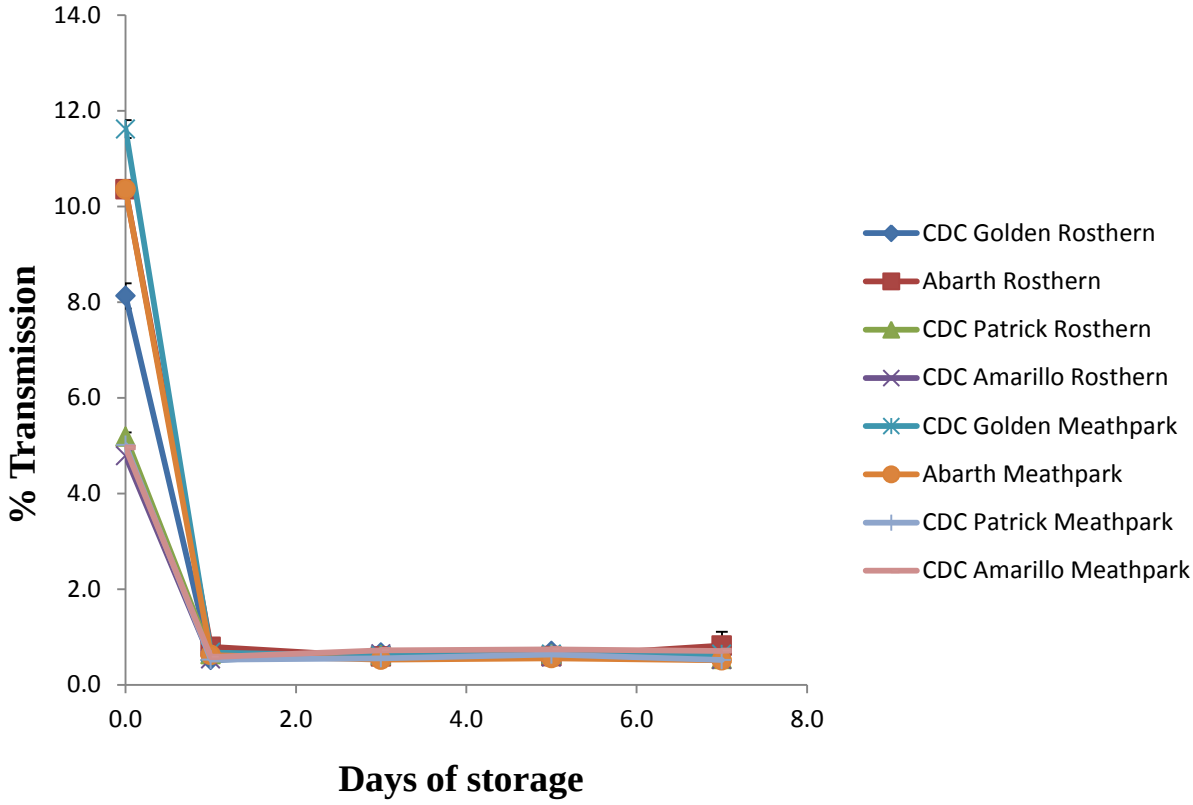

Figure 4.4: Turbidity profiles of field pea starches stored at 25°C

Chapter 5

5.1 Summary and conclusion

At both growth locations, starches isolated from the four cultivars of field peas, did not exhibit any significant ($P < 0.05$) differences with respect to chemical composition, granule morphology, SDS content and ΔH. However, the proportion of short AP chains (DP 6-12), degree of AP branching, molecular order, T_o, T_p, T_c, T_c-T_o, pasting temperature, RC, and RS content were higher in CDCG and ABAR. Whereas, the proportion of long AP chains (DP 13 – 26), thermal stability, RDS content and the final viscosity attained during pasting were higher in CDCP and CDCA. DSC and AML data revealed AM-AP co-crystallization in granules of CDCG and ABAR. Differences in structure and physicochemical properties between CDCG and ABAR and between CDCP and CDCA at both growth locations were marginal. Differences in physicochemical properties among cultivars were mainly influenced by the interplay among AP chain length distribution, degree of AP branching, molecular order, and AM-AP co-crystallization. This study showed that CDCA would be the better choice for incorporation into foods subjected to sterilization and shear (due to a lower extent of viscosity breakdown), whereas CDCG with a lower RDS and higher RS level may be a healthier choice. However, due to the high degree of set-back, all starches need to be extensively modified before they could be incorporated into foods subjected to repeated freeze-thaw cycles. Research is underway on field pea starch modification.

5.2 Directions for future research

I have listed below the areas that need to be addressed in order to obtain additional insights into the structure-property relationships among field pea starches.

1. **Morphology:** The morphology of field pea starches in the study has been mainly investigated using scanning electron microscopy, bright field microscopy and polarized light microscopy. However, atomic force microscopy can be used to visualize the morphological features of starch granules at a greater resolution and also provide topographical information that cannot be obtained from scanning electron microscopy (Baldwin, Adler, Davies, & Melia, 1998).

2. **Structure:** The structure of field pea starches was investigated using methods such as HPAEC-PAD, WAXS, and ATR-FTIR. However, the amount of amorphous and double helical content should be determined by ^{13}C CP/MAS NMR in order to obtain deeper insights into the mechanism of acid and enzyme hydrolysis (Gidley & Bociek, 1985).

3. **Retrogradation:** In this study, the progress of retrogradation was monitored using turbidity measurements (mainly measure interaction between amylose chains). However, additional methods such as DSC (provides information of amylopectin crystallization) and ^{13}C CP/MAS NMR (elucidates changes in water mobility) can also be used, since each of the above techniques measures a different property of the retrograded starch gel (Karim, Norziah, & Seow, 2000).

4. **Enzyme hydrolysis:** Studies on enzyme hydrolysis have been mainly focussed in understanding the reactivity of starches with varying amylose contents towards porcine pancreatic amylase (PPA). In this study, RDS, SDS and RS contents were determined based on the rate of glucose release. However, to gain additional insights into the kinetics of enzyme hydrolysis, the degradation pathway of PPA into the granule structure of field pea starches needs to be examined by imaging techniques such as confocal laser scanning microscopy, scanning electron microscopy and polarized light microscopy (Hoover & Zhou, 2003).

5. Acid hydrolysis: In this study, the rate and extent of acid hydrolysis of field pea starches were observed by treating native starch granules with hydrochloric acid for various time intervals. However, the structure and properties of the acid-hydrolyzed residues at different time periods of hydrolysis needs to be investigated in order to understand how starch-chain organization within the amorphous and crystalline domains influence the accessibility of H_3O^+ into the granule interior (Hoover, 2000).

6. Modification: This study has shown that the field pea starches cannot be utilized in the food industry without prior modification. Thus, modification of starch by physical and/or chemical means must be carried out in order to improve the functionality of field pea starches (Kaur, Ariffin, Bhat, & Karim, 2012).

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1. R.Raghunathan, R.Hoover, R.Waduge, Q. Liu, T.D.Warkentin. 2016. Impact of molecular structure on the physicochemical properties of starches isolated from different field pea (*Pisum sativum*) cultivars grown in Saskatchewan, Canada. *Food Chemistry*. (in review).

Conference presentations

1. R.Raghunathan, R.Hoover, V.Vamadevan, R.Waduge, Q. Liu, T.D.Warkentin (2015). A study of the structure of field pea starches at different levels of granule organization and their properties. Starch Round Table Conference, Minneapolis, Minnesota, USA, October 15-17, 2015.

Awards

1. Graduate fellowship, Memorial University of Newfoundland, St.John's, NL, Canada (from January 2014 to December 2015).

APPENDIX A

Standard curves

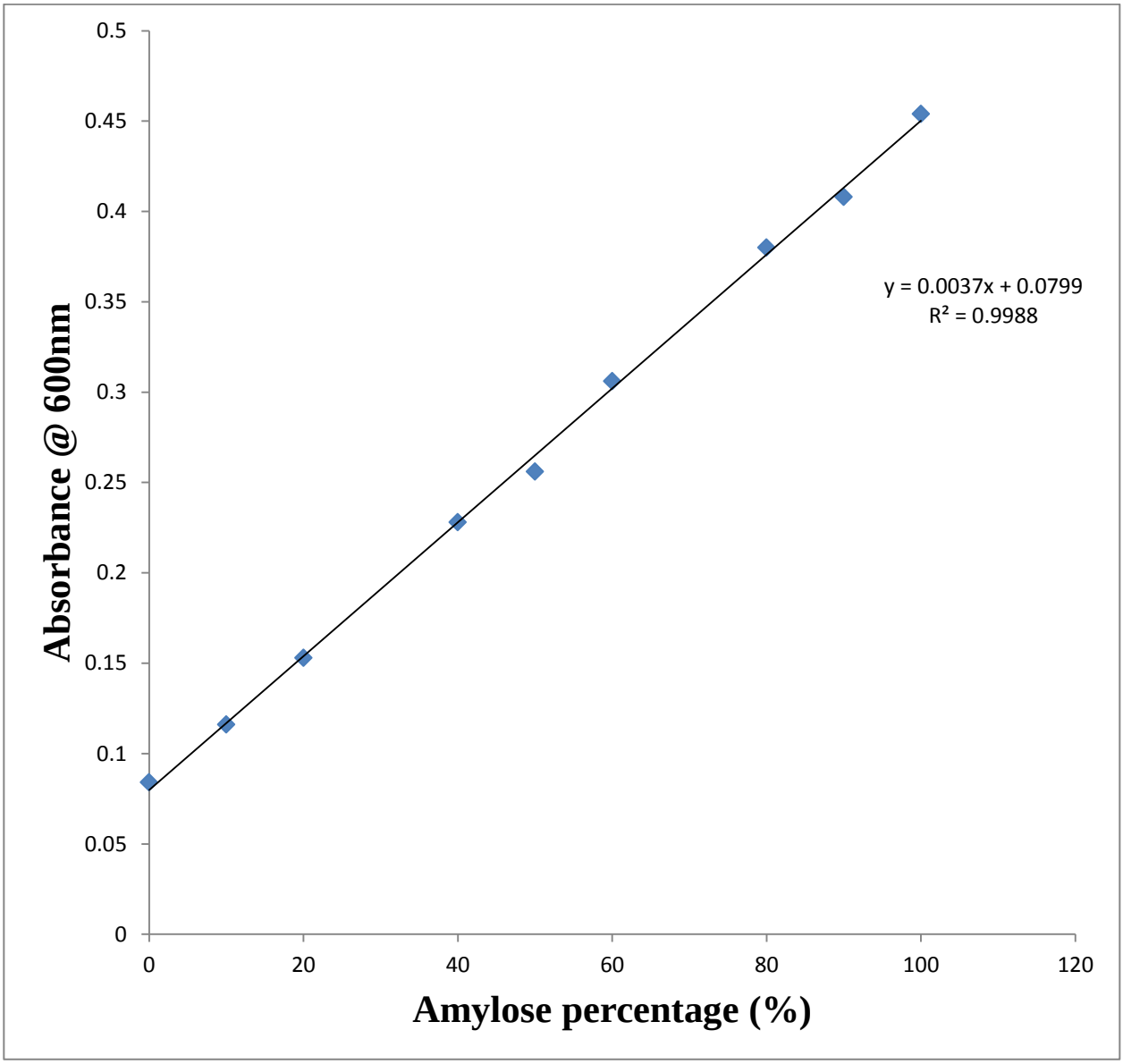

Figure A1: Standard curve for the determination of amylose content

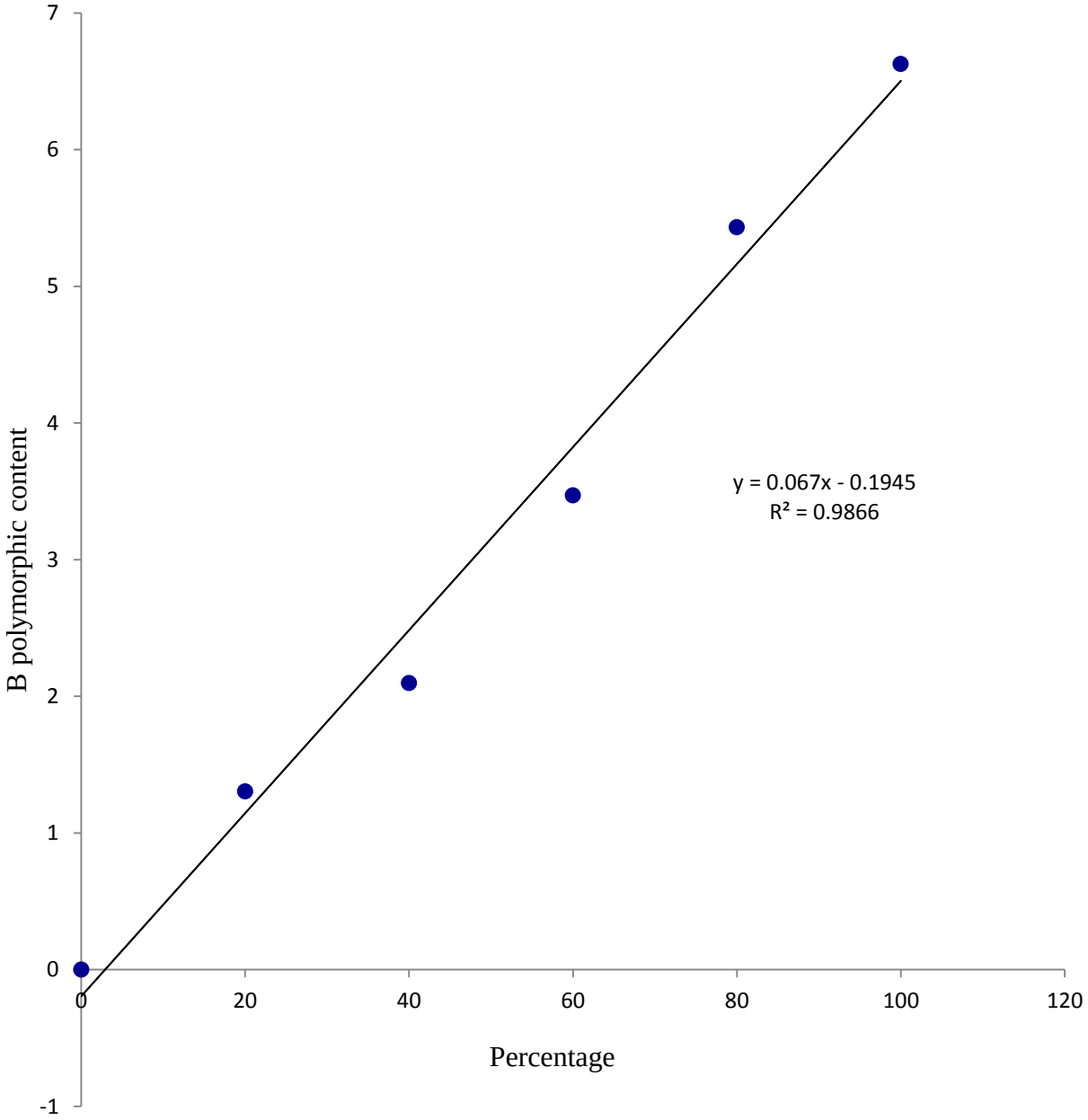

Figure A2: Standard curve for the determination of B polymorphic content

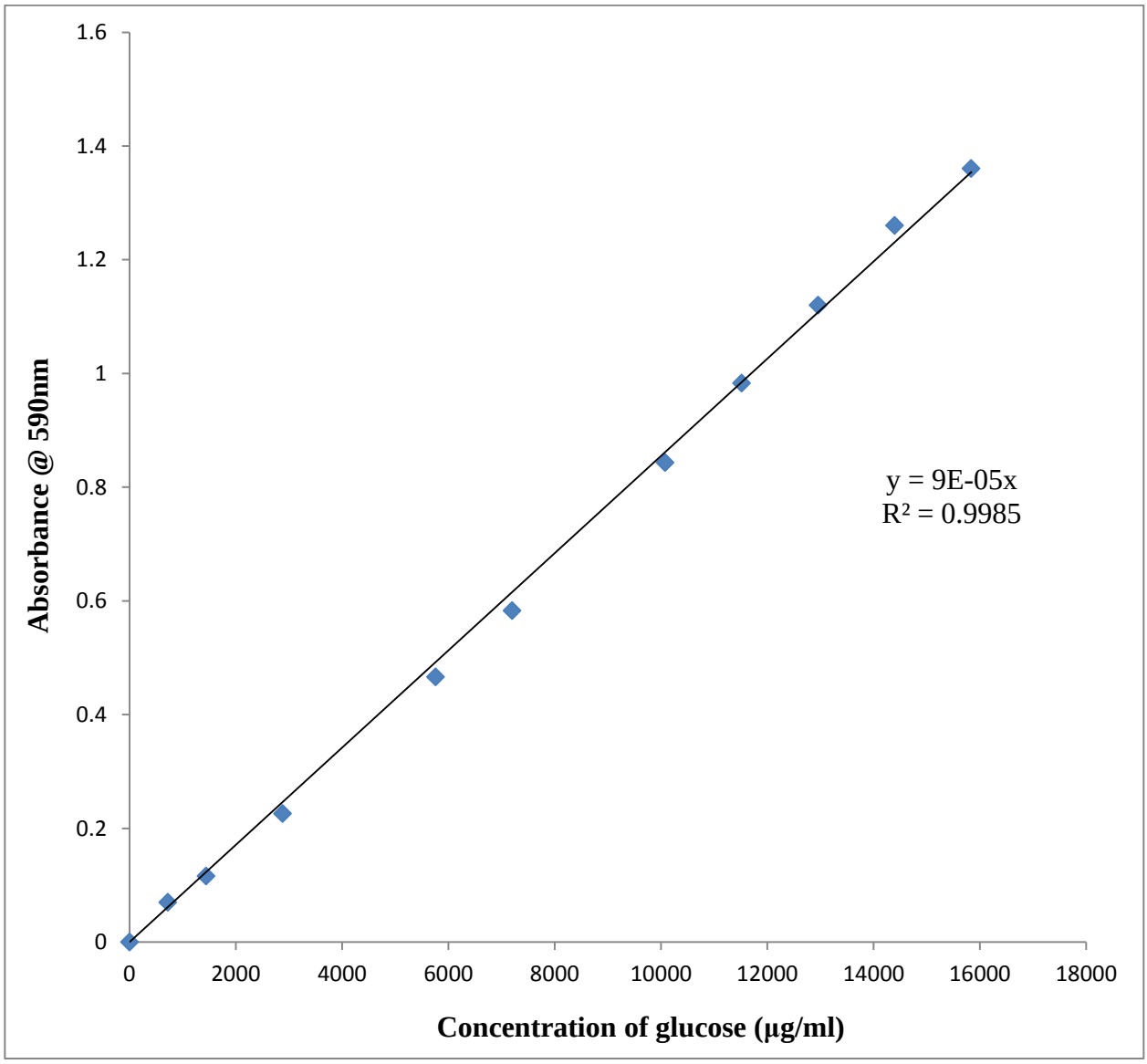

Figure A3: Standard curve for the determination of reducing sugar as glucose

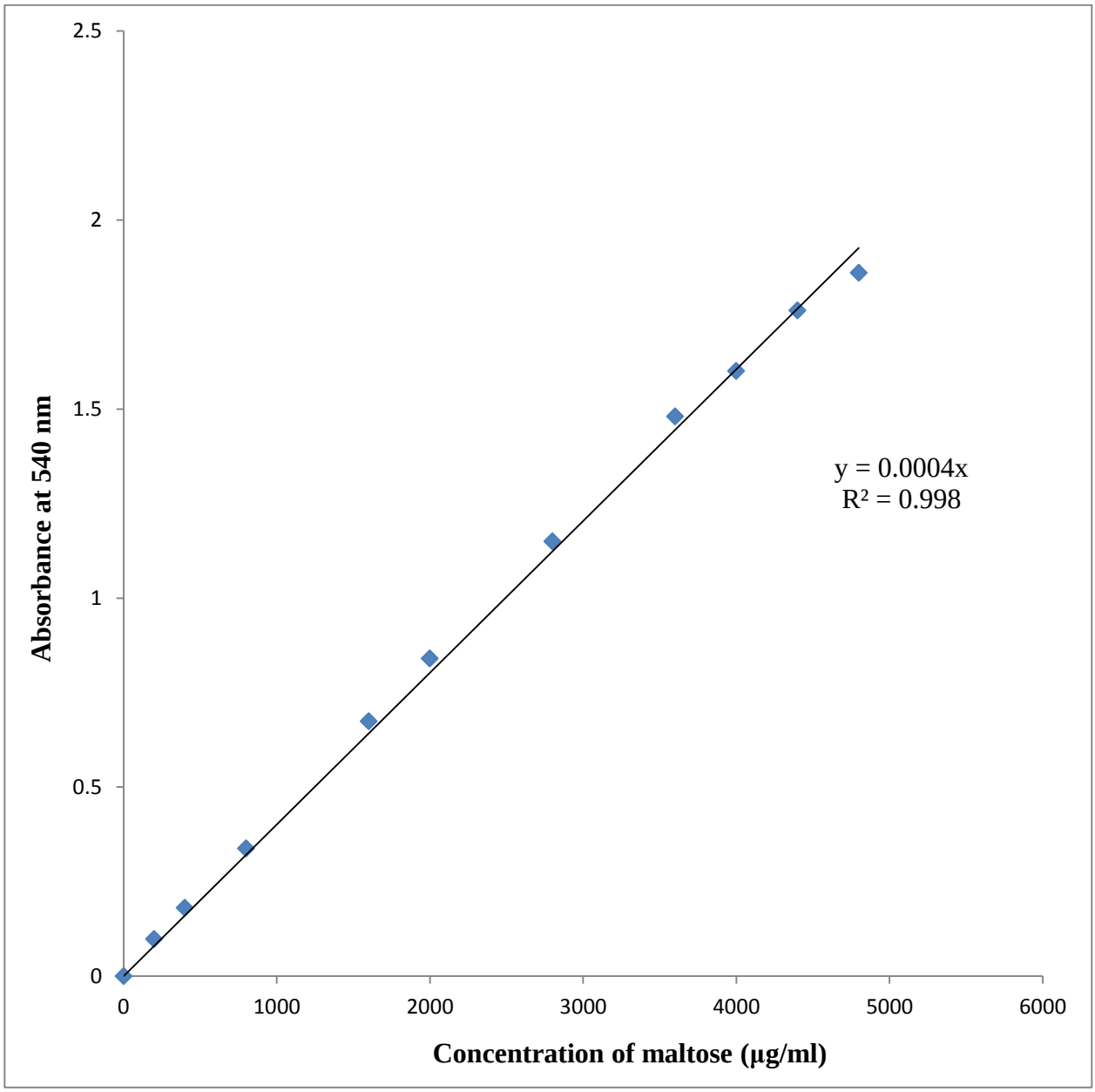

Figure A4: Standard curve for the determination of reducing sugar as maltose