COMPOSITION, MORPHOLOGY, MOLECULAR STRUCTURE, AND PHYSICOCHEMICAL PROPERTIES OF STARCHES FROM CHICKPEA (CICER ARIETINUM L.) CULTIVARS GROWN IN CANADA

TARA GISELLE HUGHES

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by

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Abstract

Starch from four chickpea cultivars grown in Canada was isolated and variability in composition, morphology, molecular structure and physicochemical properties was evaluated. The starch granules were oval to spherical with smooth surfaces. The granule size distribution ranged from 5 to 35 μm. The free lipid, bound lipid, total amylose and the amount of lipid-complexed amylose ranged from 0.04 to 0.08%, 0.21 to 0.46%, 33.9 to 40.2% and 9.1 to 15.9%, respectively. Chickpea starch had C-type X-ray pattern with relative crystallinity in the 31.3 to 34.4% range. Swelling factor of starches ranged from 1.6 to 25.9, whereas amylose leaching negligible from 50 to 70°C, ranged between 8.6 to 36.1%, at higher temperature range (50-90°C). All four starches exhibited nearly identical gelatinization temperatures. The starches differed significantly with respect to peak viscosity, breakdown viscosity, and setback. Turbidity of all starches increased steeply during the first 48 hours of storage, with only marginal increase thereafter. The amount of rapidly digestible, slowly digestible and resistant starch contents ranged from 10.9 to 15.7%, 48.5 to 60.2% and 24.1 to 40.6%, respectively.

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List of Abbreviations

α – Alpha
°C – Degrees Celsius
ΔH – Enthalpy of gelatinization
Θ -Theta
μm – Microns
μL - Microliters
Å - Anstrom
A_a – Amorphous area
AACC - American Association of Cereal Chemists
A_c – Crystalline area
ACS – American Chemical Society
AML – Amylose leaching
A_r – Absorbance of the reference
A_s – Absorbance of the sample
BU – Brabender unit
BVA – Brabender Visco-amylograph
$CaCl_2$ – Calcium chloride
C-H – Carbon to hydrogen bond
$\overline{CL}$ - Average chain length
cP – Centipose
CSLM - Confocal scanning laser microscopy
Da –Daltons
db - Double
DM – Dry mass
DMSO – Dimethyl sulfoxide
DP – Degrees of polymerization
DSC – Differential scanning calorimetry
FTIR – Fourier transform infrared spectroscopy
FW – Free water
g – Gram(s)
g/g – Grams per grams
GOPOD – Glucose oxidase-peroxidase reagent
ha –Hectares
I_2 – Iodine atom
I^{3-} - Triiodide ion
I^{5-} - Pentaiodide ion
FAO – Food and Agriculture Organization of the United Nations
g – Gram
G – Mass of glucose
GI – Glycemic index
H_3O^+ - Hydromium ion

HCl – Hydrochloric acid
^{1}H-NMR – Proton nuclear magnetic spectroscopy
HPAEC-PAD – High pressure anion exchange chromatography with pulsed amperometric detection
J/g – Joules per gram
kDa – Kilodalton
KI – Potassium iodide
kV - Kilovolt
M –Molarity
mA - Milliampere
mg – Milligram
min - Minute
mL – Milliliter
mM - Millimolarity
mPa/s – Megapascale per second
N - Normality
NaOH – Sodium hydroxide
nm - Nanometers
NMR- Nuclear magnetic resonance
RDS – Rapidly digestible starch
rpm – Revolutions per minute
RS – Resistant starch
RVU – Rapid visco analyser unit
RVA – Rapid visco-analyser
s - Second
SAXS - Small angle X-ray scattering
SDS – Slowly digestible starch
SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM – Scanning electron microscopy
SF – Swelling factor
SP Swelling power
T_c - Conclusion temperature
T_c-T_o - Gelatinization temperature range
T_o - Onset temperature
UV – Ultraviolet
V_o – Initial volume of starch
V_1 – Initial volume of water
V_2 – Volume of the swollen granule
v/v – Volume per volume
v/v/v – volume per volume per volume
W - Weight
WAXD - Wide angle X-ray scattering and diffraction
w/v – Weight per volume
w/w – Weight per weight

Chapter 1: Introduction

Legumes are the dicotyledonous seed of plants that belong to the Leguminosae family (Hoover and Sosulski, 1991) which have 16 000-19 000 species in approximately 750 genera (Ratnayake et al., 2001). Legumes are consumed whole as food in many parts of the world because of their high protein content (20-50%) compared to other crops (Singh, Sandhu, and Kaur, 2004). This makes legumes a significant food source for developing countries, low-income people (Bressani and Elias, 1979) and even agricultural feed, with chickpea ranking fifth in nutritional importance worldwide (Singh, Sandhu, and Kaur, 2004).

Legumes are also classified as resistant starches which are digested slowly, have a low glycemic index (GI), and are fermented in the large intestine to produce short-chain fatty acids (Sandhu and Lim, 2008) which are beneficial for colon health and may be protective against colonic cancer (Han et al., 2006), as well as providing other health benefits. However, most legumes grown in Canada, with the exception of peas, are exported due to low national consumption and marginal secondary processing applications. This is because, legume starches have very few commercial applications due to their restricted swelling power, poor granule dispersibility, high gelatinization transition temperatures, high extent of water exudation (syneresis) and resistance to enzyme hydrolysis (Hoover et al., 2010).

Starch is the most abundant carbohydrate in the legume seed, accounting for 22-45% of the seed (Hoover and Ratnayake, 2002). Extensive research on cereal, potato,

sweet potato, and cassava starches makes them readily available for use in food and non-food applications, whereas there is a dearth of information on structure-property relationships among pulse starches (Hoover and Ratnayake, 2002).

The overall objective of this study was to determine structure-property relationships among four newly introduced chickpea cultivars grown in Canada at the same location and under the same environmental conditions. Recognition of inherent genetic variation in starch properties among cultivars could be useful for plant breeders, who may wish to develop or select potentially useful cultivars with certain functional properties of their starches. The results of this study would form the basis for further investigations on chemical and physical modification to improve the functional characteristics of the above.

Chapter 2: Literature Review

2.1 General Information and Utilization

2.1.1 Starch

Starch is a polysaccharide that is produced by all photosynthetic plants, and used as an energy store. Currently, there are many food and non-food applications for starch. For instance, starch is a major component of the diet and is used universally in food systems, which is primarily governed by its gelatinization, gelation, pasting, solubility, swelling and digestibility properties (Brown, 2004; Singh et al, 2004). Because of those properties, starch is considered to be commercially important because of its high demand as an ingredient for a variety of processed foods (Singh, Sandhu and Kaur, 2004, Kaur; Singh, and Sandhu, 2004). These include canned goods, cereals and snack foods, baked goods, soups, dressings, and as a meat binder. Starches can also be used industrially in non-food applications. These include their numerous applications in the adhesive, paper, mining, textile, construction, metal, cosmetic, and pharmaceutical industries, to name a few.

There are three predominant classes of starches used for food applications, although it is more or less only starch and starch derivatives of maize and potato that have vast commercial interest currently (Lawal and Adebowale, 2005). These classifications of starches include cereal starches such as wheat, rye and barley, maize,

and rice, tuber and root starches such as potato, sweet potato, cassava, and yam, and legume starches, in which the latter is classified into two groups based on their seed compositions. In this case, the first group includes legumes such as soybean and peanut, in which the seeds comprise essentially protein (25-40% by weight) and lipid (20-25%) with only minimal amount of starch (less than 1%), whereas in the second group, also known as pulse starches, starch is the main component (30-40%; Yoshimoto et al., 2001).

2.1.2 Pulses

Pulses are defined by the Food and Agriculture Organization (FAO) of the United Nations as annual leguminous crops yielding from 1 to 12 grains or seeds of variable size, shape, and color within a pod (Chung et al., 2008b). Pulse crops are part of the leguminosae family but pulse refers to only the dried seeds which primarily include four major types, dried peas, bean, lentils and chickpea **(Figure 2-1),** constituting about 60 domesticated species worldwide (Hoover et al., 2010).

Pulses come in a variety of shapes, sizes and colors and can be consumed in many forms including whole or split, ground flours or separated into fractions such as protein, fiber and starch (www.pulsecanada.com). Therefore, pulse starches can have numerous applications including human consumption, livestock feed, as well as other uses. For the most part, lentils, beans and chickpea are primarily used for human consumption, with dry pea to a lesser extent. Pulses are a great addition to any diet since they are great

Figure 2-1: Classification of legume starches

Adapted from Pulse Canada website with permission


Legumes
Soybeans
Peanuts
Pulses
Dried Beans
Dried Peas
Chickpeas
Lentils
Fresh Peas
Fresh Beans

source of valuable nutrients. In fact, pulses are an important part of the human diet in many parts of the world because they are a rich and inexpensive source of protein (20-50%; Jood, Bishnoi and Sharma, 1998; Singh, Sandhu and Kaur, 2004). They are also high in complex carbohydrates, especially fiber, resistant starch, and slowly digestible starch, as well as important vitamins and minerals such as iron, zinc, phosphorous, folate, potassium and some B-vitamins, but are known to have relatively low fat contents. These nutrients are responsible for the many health benefits that are positively associated with pulse consumption, including prevention and/or risk reduction of cardiovascular disease, diabetes, and several forms of cancers. Pulses are also considered a good food source for weight management, because of their low fat, and high protein, fiber and resistant starch profiles, which cause delayed gastric emptying, resulting in reduced hunger, early feeling of fullness, and increased satiety after a meal (Schneeman, 2002).

Aside from human nutrition, pulse seeds are also important in animal nutrition because they contain high amounts of essential amino acids and have high protein contents (20-50%), which is twice the level found in cereal starches and significantly higher than tuber starches (Singh, Sandhu and Kaur, 2004). In particular, in Canada, dry peas, is widely used for livestock feed, especially in the hog industry, with lesser amounts used for cattle and poultry feed, while low grade chickpeas and residual beans and lentils are also used to a lesser extent (www.agr.gc.ca).

2.1.3 Chickpea

Chickpea (*Cicer arietinum* L.) is an ancient pulse crop first grown in Turkey about 7,000 B.C. Chickpea contains on average 17% protein, 5.3% fat, 3.9% fiber and 70% carbohydrate, in which a major proportion is starch, accounting for 37.5-50.8% (Sayar, Koksel, and Turhan, 2005; Singh, Sandhu and Kaur, 2004). Because of its high protein content, chickpea is widely consumed in developing countries as a high source of protein, while in developed countries, it is considered more of a health food (Goodwin, 2003).

Figure 2-2 illustrates the two main types of chickpea, the kabuli and desi types, both of which have different varieties or cultivars. The kabuli variation of chickpea, also known as garbanzo beans, are larger in size and characterized by a cream-colored seed, thin seed coat and colored flowers. The desi variation, however, are smaller in size, have darker colored seed, a thick seed coat, and white flowers. The desi type of chickpea accounts for about 80-85% of the total chickpea area and are predominantly grown in Asia and Africa, while the Kabuli types are grown in West Asia, North Africa, North America, and Europe (Pande et al., 2005). However, the kabuli chickpea is known to be nutritionally better than desi chickpea because of a higher amount of utilizable proteins and its high biological value (Jood, Bishnoi and Sharma, 1998). They also contain more carbohydrates but a lower fiber content than the desi cultivars (Chavan et al., 1986). Both chickpea types are primarily consumed whole in salads, soups and so on. However, the desi type can also be split or milled into flour, known as besan, which is commonly used commercially in snack foods.

Figure 2-2: Two variations of chickpea seeds: A) kabuli and B) desi

Adapted from Pulse Canada website with permission

A

B

As with other pulses, consumption of chickpea also provides many health benefits, so as it could be considered a functional food. This means that chickpea can be classified as a healthful product, which can provide benefits beyond those rendered by traditional food, or a food that, because of the presence of physiologically active components, can provide health benefits beyond basic nutrition (Hasler, 2002; Milner, 2000). These benefits are likely because chickpea is known to be an excellent source of vitamins, minerals, and dietary fiber (Jukanti et al., 2012).

Because of its high fibre content, chickpea is known to reduce cardiovascular disease and diabetes. Chickpea, contains higher amounts of resistant starch and amylose, which release glucose into the bloodstream slowly, thus, it can help reduce cholesterol and regulate blood sugar, both of which help in the prevention of cardiovascular disease and diabetes, respectively (Jukanti et al., 2012). Moreover, reports show that chickpea has a relatively low glycemic index of 55 or less, which makes it helpful in controlling blood glucose levels in type-2 diabetic patients (www.agr.gc.ca).

Chickpea consumption is also beneficial for those with modified diets. For example, chickpea flour is gluten-free, and thus suitable for individuals afflicted by celiac disease. Likewise, the high protein and iron content of chickpea makes it a wise choice for consumption in vegetarian diets. In addition, consumption of chickpea during pregnancy can also have benefits, since it is an excellent source of folate, a vitamin that reduces neural tube defects in a developing embryo.

Finally, chickpea consumption may also be positively correlated with decrease risk of cancer. In particular, the high fiber content of chickpea can help lower the risk of

colon cancer, as well as have a positive effect on weight loss. Likewise, studies have shown that increased amounts of the folate vitamin can also lower the chances of colon and cervical cancer (www.agr.gc.ca).

2.2 Production

2.2.1 Pulse Production

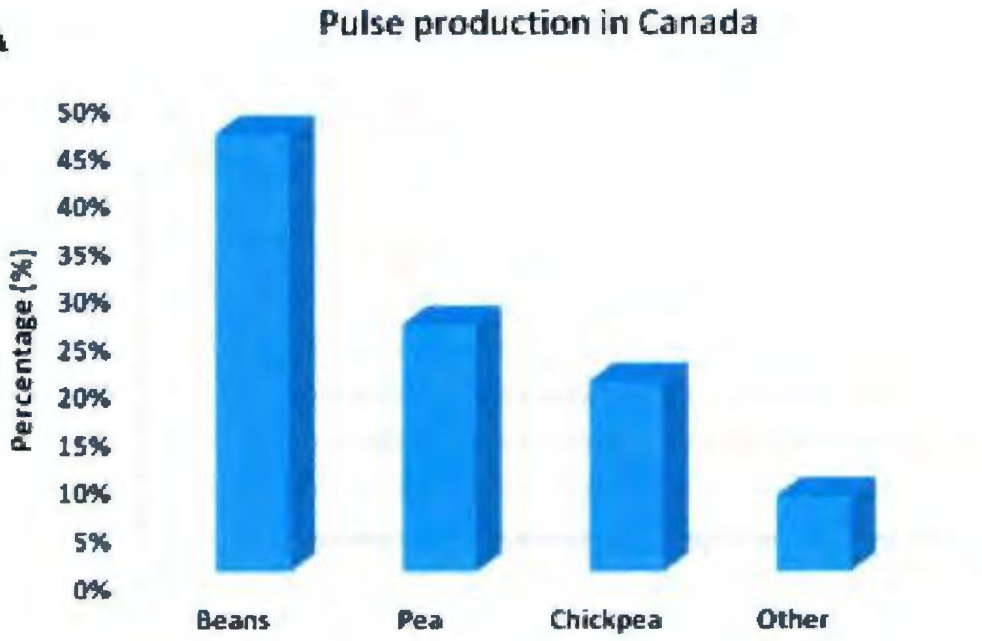
Global production of pulses is approximately 70.4 x 10^6 tons per year (FAO, 2012), with India being the largest producer, followed by Canada, Brazil, and China as is shown in **Figure 2-3**. In fact, India is both the largest producer and consumer of pulses in the world, accounting for 23% (16.28 x 10^6 tons) of the global production in 2012 (FAO 2012).

Pulse production in Canada is a rapidly growing industry, in which Canada now accounts for approximately 35% of global pulse trade each year (www.pulsecanada.com). Canada is the second largest producer of pulses, ranking first in worldwide production of peas, second in lentils and ninth in chickpeas (**Figure 2-3**) worldwide (Hoover et al., 2010). Pulse production in Canada peaked in 2010, producing more than 5.7 million tons, with pulse production normally in the range of 4.5 to 5 million tons per year, from about 1,000,000 tons in the early 1990s (www.pulsecanada.com; Hoover and Ratnayake, 2002). Canada's major pulse crops are beans, followed by pea, chickpea, and lentil (**Figure 2-3**).

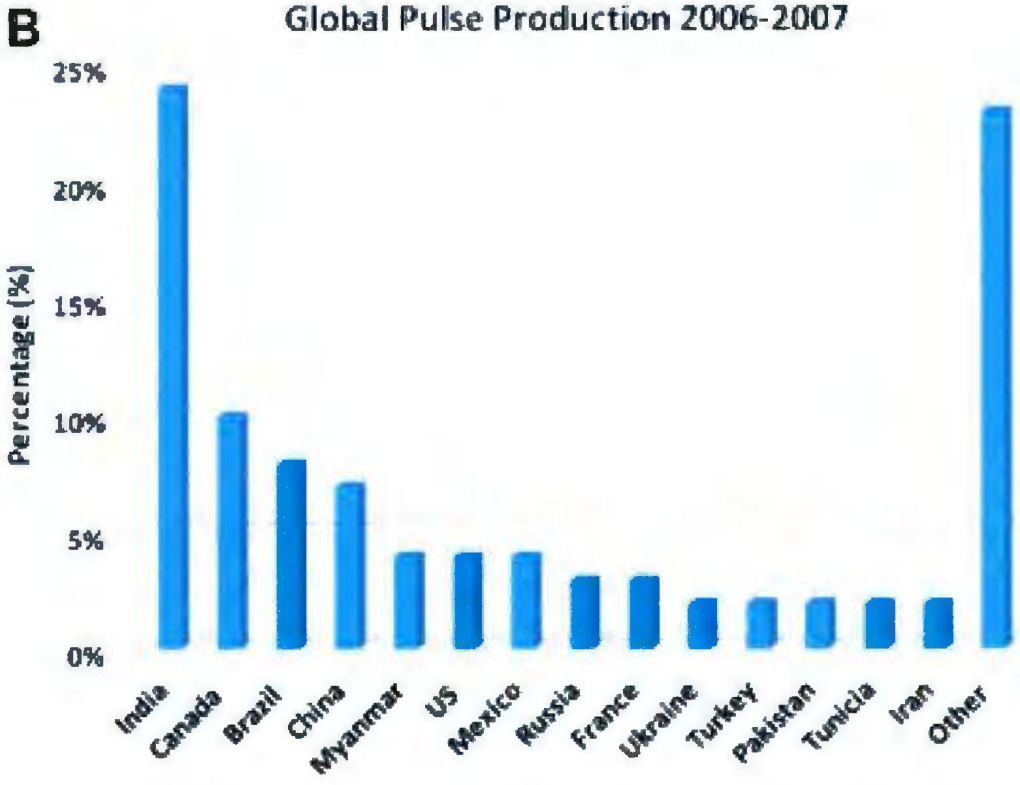
Figure 2-3: Pulse production: A) Canada 2006-2007, and Global 2006-2007

Adapted from Hoover et al. (2010) with permission from Elsevier

A


Pulse production in Canada
Percentage (%)
50%
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
Beans
Pea
Chickpea
Other

B


Global Pulse Production 2006-2007
Percentage (%)
25%
20%
15%
10%
5%
0%
India
Canada
Brazil
China
Myanmar
US
Mexico
Russia
France
Ukraine
Turkey
Pakistan
Tunicia
Iran
Other

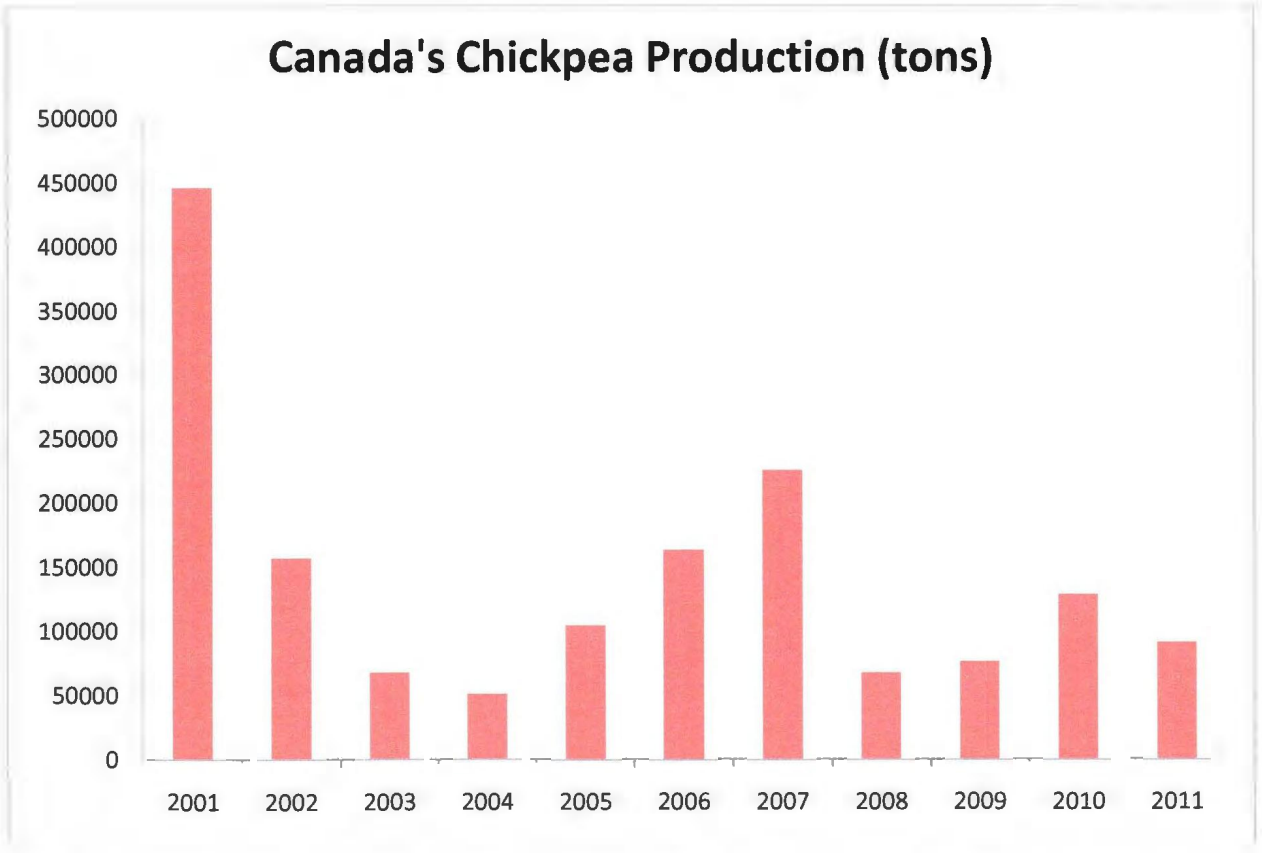
2.2.2 Chickpea Production

Chickpea is the third most important food legume grown in the world, after beans and peas, with approximately 11.3 m ha with 9.6 million tons production (FAO, 2011; Goodwin, 2003; Kaur and Singh, 2006). Chickpea was traditionally grown in semi-arid zones of India and Middle Eastern countries (Goodwin, 2003). Currently, chickpea is grown in over 50 countries across the Indian subcontinent, North Africa, the Middle East, southern Europe, the Americas and Australia (Jukanti et al., 2012; Singh et al., 2008).

Production of chickpea in Canada over the last 10 years is illustrated in **Figure 2-4**. The majority of chickpea that is grown in Canada is exported to other markets, mainly the US, Middle East and India. Although, exports of pulse crops as a whole have significantly increased in recent years, Canadian chickpea exports declined 10% from nearly $56 million in 2006 to almost 50 million dollars in 2009 (www.agr.gc.ca). This export market is forecasted to increase in Canada for the year 2012-2013.

In Canada, chickpeas are grown in the cool climatic soils of southern Alberta and Saskatchewan, where about 96% of chickpea production occurs in Saskatchewan and only 4% in Alberta. The production of chickpea in Canada are evenly split between desi (50%) and kabuli (50%) types; with kabuli and desi best adapted to the brown and dark brown soil zones, respectively of Saskatchewan (Goodwin, 2003). However, chickpea is not well adapted to saline soils, soils with high clay content, soils that are slow to warm up in the spring, or to high moisture areas since it will not tolerate waterlogged soil (Goodwin, 2003). Globally, the desi type accounts for close to 80% of chickpea

Figure 2-4: Chickpea production in Canada, 2001-2011 (FAO)


Canada's Chickpea Production (tons)
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011

production, most likely because it can withstand cooler temperatures and matures quicker than kabuli chickpea (www.agr.gc.ca).

There are several setbacks to chickpea farming in Canada. The largest of these setbacks would be the ascochyta blight, a devastating seed and residue-borne fungus, whose host is the chickpea with an approximate 4 year cycle (Goodwin, 2003). Chickpea production in Canada is also affected by the length of growing season. Ideally, chickpea requires a long growing season, thus limiting their growth in Canada. Under ideal conditions, the growing season in Canada is long enough providing there are no setbacks such as late seeding, slow germination, disease, wind damage, and early fall frost (Goodwin, 2003). A further concern is that chickpea has an indeterminate growth habit, meaning that flowering and pod filling will continue either simultaneously or alternatively until the temperature and moisture permits. Thus, if the plants are immature when the growth conditions change, there could be increased amount of immature seeds in the crop, which decreases quality (Goodwin, 2003).

2.3 Starch Composition and Structure

2.3.1 Major Components

The two major components of starch are amylose and amylopectin which make up approximately 98-99% of the dry weight. The relative proportions of each of the major polysaccharides vary according to the starch botanical source, species, and cultivar,

where most species contain approximately 25% amylose and 75% amylopectin (Tester et al., 2004; Eliasson and Gudmundsson; 1996, Gailliard and Bowler; 1987). Amylose and amylopectin have different properties in which amylose has a high tendency to retrograde and produce tough gels and strong films, while amylopectin, when dispersed in water, is more stable and produces soft gels and weak films (Pérez and Bertoft, 2010). In general, pulse starches are characterized by high amylose contents (**Table 2-1**), ranging from 19.5 to 75.4% (Hoover and Sosulski, 1985; 1991).

2.3.1.1 Amylose

Amylose is a relatively long linear α-glucan (**Figure 2-5A**), but also contains a few branch points. That is, amylose is composed of approximately 99% α-(1→4)-D-glucose linkages, and 1% α-(1→6)-D-glucose linkages, producing 9-20 branch points per molecule, which is equivalent to 3-11 chains per molecule (Hizukuri et al., 1981; Tester and Karkalas, 2001; Yoshimoto et al., 2000). The extent of branching depends on amylose origin and increases with molecular size of amylose (Biliaderis, 1998). The branched amylose molecule is suggested to have an intermediate structure between the linear amylose and amylopectin and thus is often referred to as intermediate material (Takeda, Tomooka, and Hizukuri, 1993).

The molecular weight and size of amylose generally ranges from $1x10^5$ to $2x10^6$ Da, and 200 to 20,000 degrees of polymerization (DP), respectively (Tester and Karkalas, 2001). The DP values of legume starches are generally lower than those of tuber and root

Table 2-1: Lipid and amylose contents of pulse starches

Starch Source	Lipid (%)			Amylose (%)			Lipid-Complexed Amylose (%)
	Total[4]	Surface[2]	Bound[3]	Unspecified	Apparent	Total	
Chickpea[1,d,h,e]	0.10-0.50	0.6-0.9	-	27.2	10.8-21.2	23.0-23.3[3]	9.0-10.0
Beach Pea[c]	0.16	0.06	0.10	-	27.3	29.02	5.9
Black Bean[e,k]	0.20-0.40	0.08-0.10	0.26-0.43	-	23.2-35.2	27.2-39.3[3]	10.37-14.9
Cowpea[h]	0.15	-	-	25.8	-	-	-
Field Pea[j]	-	0.05	0.24-0.29	-	42.9-43.7	48.8-49.6[3]	10.9-12.3
Grass Pea[i,c]	0.12	0.04-0.05	0.08	-	34.52	36.4-38.3[3]	-
Green Pea[c]	0.19	0.07[2]	0.12	-	32.67	36.70	11.0
Jack Bean[a,b]	0.14	-	-	-	37.5	-	-
Lentil[d,e,k]	0.30-0.40	0.01-2.0	0.3-0.81	-	13.1-28.8	23.5-32.3[3]	6.0-10.9
Lima Bean[a]	0.54	-	-	32.7	-	-	-
Mung Bean[f]	-	0.04	0.27	-	39.8	45.3[3]	12.1
Navy Bean[e,k]	0.30	-	-	-	26.0-26.1	28.2-28.6[3]	7.8-8.7
Pigeon Pea[g]	0.13	0.03	0.10	-	28.5	29.3	2.7
Pinto Bean[e,k]	0.50-0.55	0.04-0.06	0.43-0.57	-	27.8-30.1	31.3-35.5[3]	11.8-15.7
Smooth Pea[e,k]	0.3-0.4	0.02-0.03	0.47-0.48	-	22.0-31.0	23.9-35.1[3]	7.9-11.8
Velvet Bean[b]	0.4	-	-	39.2	-	-	-
Wrinkled Pea[k]	-	0.05	0.80	-	68.8	78.4[3]	12.2
Yellow Pea[h]	0.07	-	-	31.2	-	-	-

[1]Letter superscript indicates the reference: [a]Betancur-Ancona et al., 2002a, [b]Betancur-Ancona et al., 2002b, [c]Chavan et al., 1999, [d]Chung et al., 2008, [e]Hoover and Ratnayake, 2002, [f]Hoover et al., 1997, [g]Hoover et al., 1993, [h]Huang et al., 2007, [i]Jayakody et al., 2007, [j]Ratnayake et al., 2001, [k]Zhou et al., 2004.

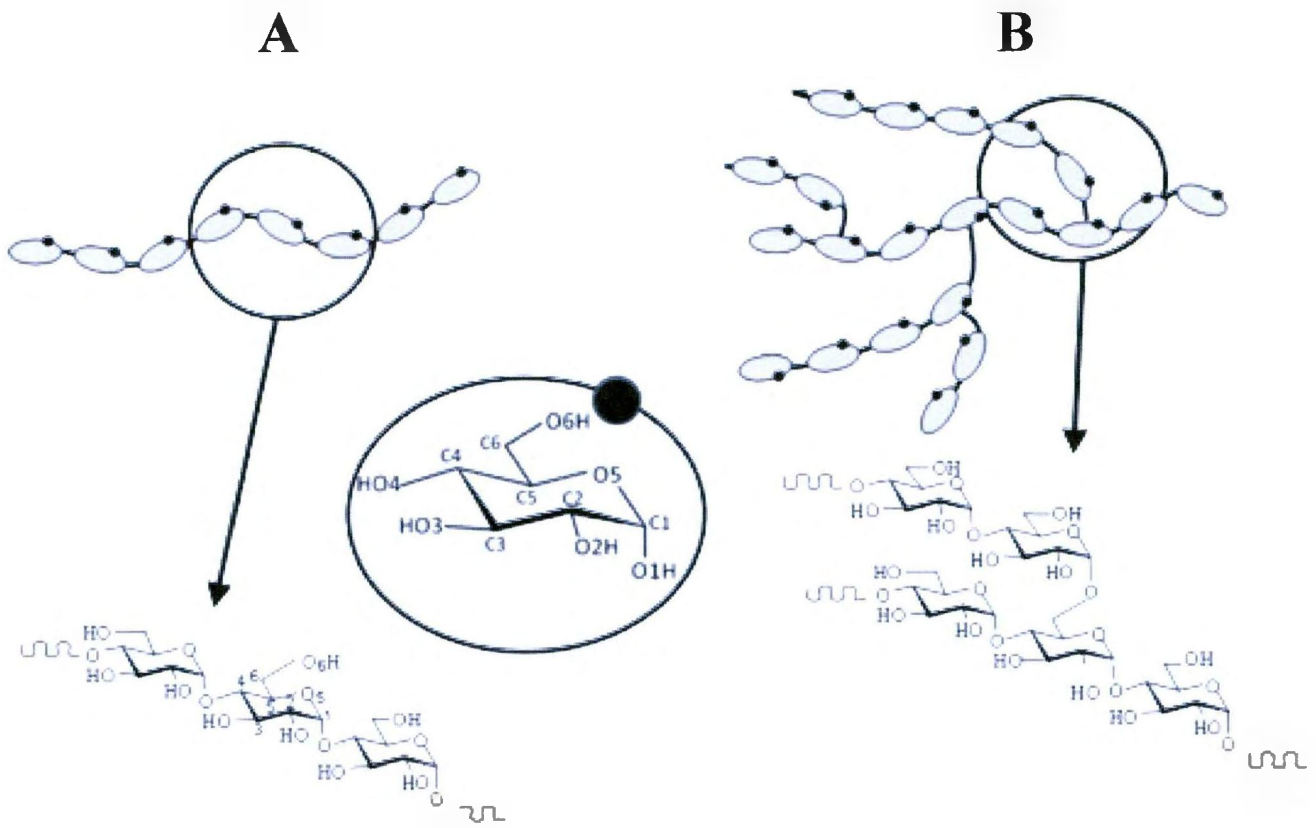
[2]Lipids extracted using chloroform: methanol solution (2:1 ratio).

[3]Lipids extracted using and n-propanol: water solution (3:1 ratio)

[4]Lipids extracted by acid hydrolysis of native starch

Figure 2-5: Basic structures of A) amylose and B) amylopectin

Adapted from Pérez and Bertoft (2010) with permission from John Wiley & Sons


A
B
O6H
C6
C4
HO4
O5
C5
C2
HO3
C3
O2H
C1
O1H

starches (Hizukuri, 1986). Each amylose chain contains approximately 200-700 glucose residues (Tester and Karkalas, 2002).

2.3.1.1.1 Amylose structure

Amylose is thought to be arranged in a left-handed helix due to the natural twist that is present in the glucose chair conformation (Kowblansky, 1985). The amylose helix consists of 6 glucose units per turn, with exterior and central cavity diameters of 12.97 Å and 5 Å, respectively (Fonslick and Khan, 1989). The inside of the amylose helix has 6 or 7 glucose units per turn and a diameter of 4.5-6.0 Å (Krog, 1971). The amylose helix is stabilized by hydrogen bonds between the hydroxyl groups of adjacent glucosyl resides and inter-turn hydrogen bonds located on the outer surface of the helix (Banks and Greenwood, 1975). Studies of light scattering, viscosity analysis, and molecular weight have shown that the conformation of the amylose helix in an aqueous solution appears to be either a random helical coil with 6 glucose units per turn (Banks and Greenwood, 1971), an interrupted helix, such as a segregated helix with 10-15 turns and also linear parts in the same molecule, or otherwise a deformed helix/worm-like coil (Rao and Foster, 1963). Amylose helices are characterized by a lipophilic core consisting of C-H groups and glycosidic oxygen, with the polar hydroxyl group located on the outer surface of the helix (Hoover, 1998).

In a freshly prepared aqueous solution, the amylose chain adapts an unstable random coil structure, which is unstable (Pérez and Bertoft, 2010). However, amylose tends to instantly form helical inclusion complexes with several molecules, including

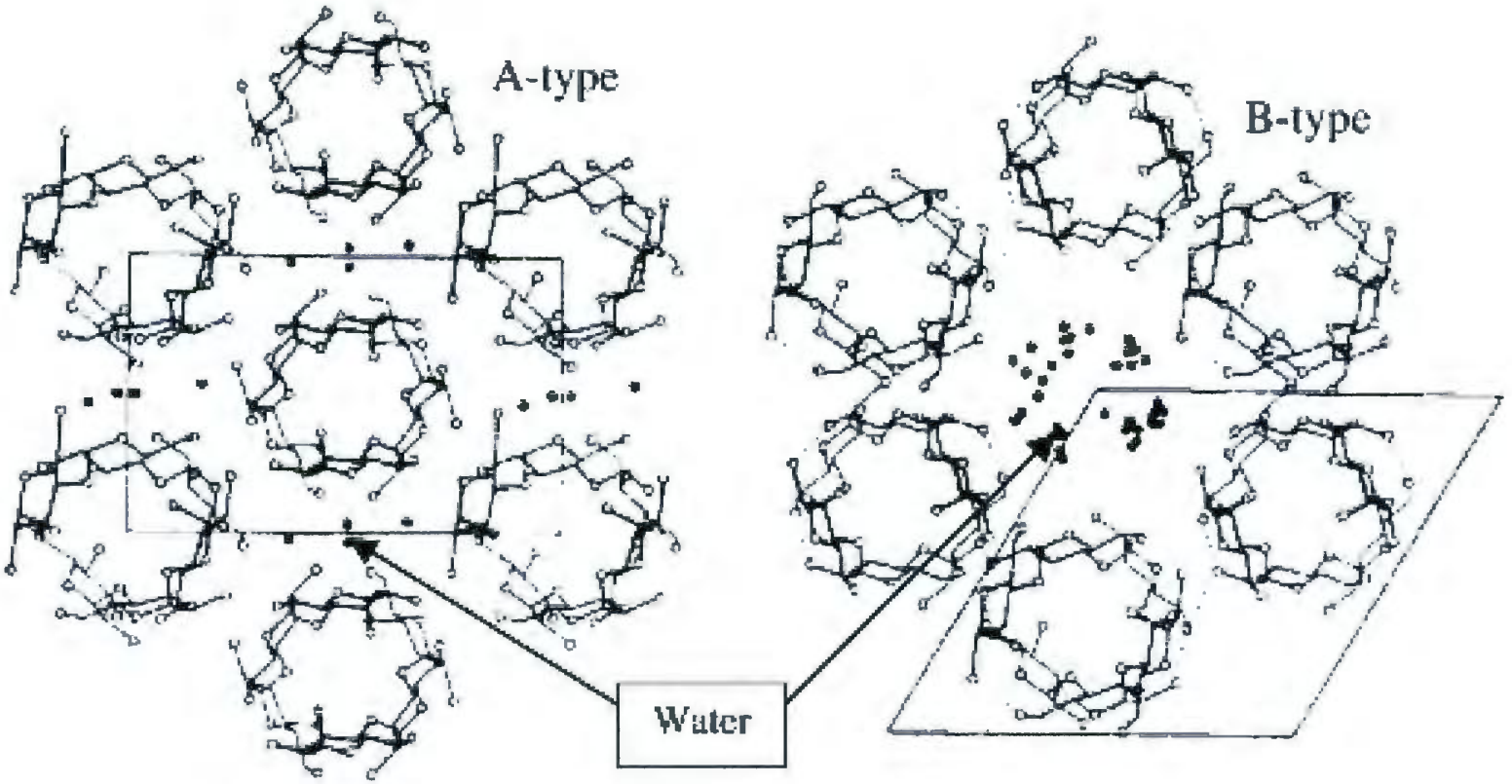
lipids, iodine, alcohols, dimethylsulfoxide, and flavor compounds. Those complexes are referred to as clathrates or helical inclusion compounds (Hosney, 1994). These compounds enable the formation of left handed amylose single helices (also known as V-amylose), which display the V-type diffraction pattern (Pérez and Bertoft, 2010). This structure of amylose commonly has 6 glucose units per turn, but maybe 7 or 8 in the presence of bulky compounds, whereby the ligands are stabilized by hydrogen bonds between the hydroxyl groups of adjacent glycosyl residues, and many inter- and intra-molecular van der Waals forces (Buléon et al., 1998; Banks and Greenwood, 1975). However, Waduge et al. (2006) reported that inclusion complexes may also be formed by trapping molecules between amylose and amylopectin fractions.

In the absence of complexing agents, amylose molecules can gradually associate to form double helices, characterized as either A- or B-type. A minimum chain length of DP 10 is required for double helix formation in a pure oligosaccharide solution, while shorter oligosaccharides can co-crystallize in the presence of longer chains (Pérez and Bertoft, 2010). A and B forms of amylose are characterized by left-handed and parallel helices with six glucose units per turn, with a repeating unit of 10.5Å (Buléon et al., 1998; Pérez and Bertoft, 2010). However, the packing of the double helices differ in the A- and B-type starches.

In the A-type starches (**Figure 2-6**), amylose chains are crystallized in a monoclinic lattice, with each unit cell containing 12 glucopyranose units packed in parallel, with four water molecules located between the helices (Pérez and Bertoft, 2010). B-type starches (**Figure 2-6**) have chains crystallized in a hexagonal lattice, with the

Figure 2-6: 3D Structures of A-type and B-type crystalline amylose polymorphs

Adapted from Wu and Sarko (1978) with permission from Elsevier


A-type
B-type
Water

double helices packed parallel with 36 water molecules, half of which are tightly bound to the double helices with the remainder forming a complex network centered around the unit cell axis (Pérez and Bertoft, 2010). This central cavity of the B-type starch is able to include molecules such as fats into its interior.

2.3.1.1.2 Amylose-lipid complexes

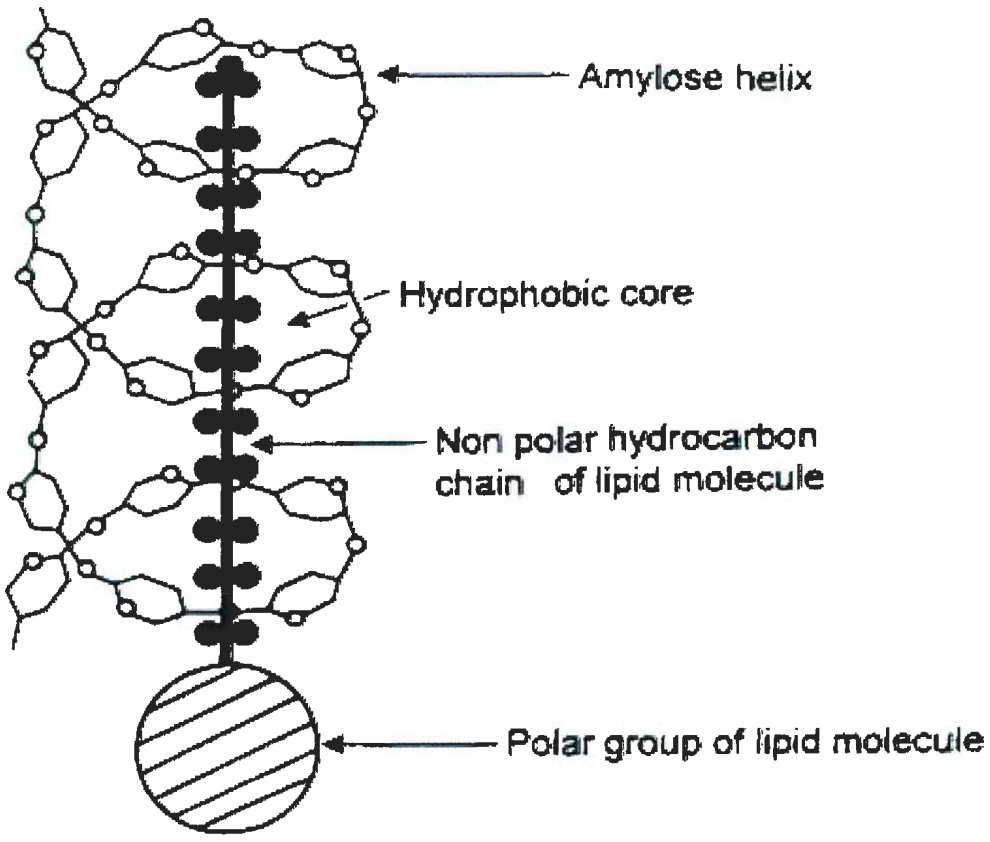
Complex formation between starch and fatty acids was first reported for corn starch (Schoch and Williams, 1944) and is known to greatly influence functional properties since it can greatly restrict the hydration capacity and granule swelling of starch (Hoover, 2001).

The formation of amylose-lipid complexes can occur during starch biosynthesis in the presence of natural fatty acids and phospholipids. Amylose-lipid complexes can also be formed during gelatinization in the presence of natural fatty acids or added ligands such as monoacylglycerides, stearoyl lactate, or sorbital monostearate.

The core of the amylose helix consists solely of C-H bonds and is thus hydrophobic (Godet et al., 1993). Consequently, when present, the hydrophobic (C-H) chain of the fatty acid is located inside the amylose helix core, stabilized by van der Waals contacts with adjacent hydrogens of amylose, while the polar ends of the lipid remain outside (**Figure 2-7**; Godet et al., 1993). The formation of amylose-lipid complexes depends on the length of the fatty acid molecule. In this case, lipids and surfactants require a minimum of 8 carbons in the fatty acid chain to form a complex (Yamamoto et al., 1984), with the optimum suggested chain length of 12 to 14 carbons

Figure 2-7: Inclusion of lipid molecule into an amylose helix

Adapted from Carlson et al. (1979) with permission from John Wiley & Sons


Amylose helix
Hydrophobic core
Non polar hydrocarbon chain of lipid molecule
Polar group of lipid molecule

(Hoover and Hadziyev, 1981). The characteristic "V" X-ray pattern of amylose-complexed lipids is reported in high amylose starches, as well as in dull or sugary starches (Zobel, 1988).

2.3.1.1.3 Amylose-iodine complexes

The production of the characteristic blue color associated with the formation of the amylose-iodine complex has some applications, such as in the measurement of amylose content and the extent of amylose leaching during heating. In fact, the reaction between amylose and iodine has been established for centuries where it was first proposed that iodine is present in a unidimensional array within an amylose helix composed of six glucose units per turn (Rundle, Foster and Baldwin, 1944).

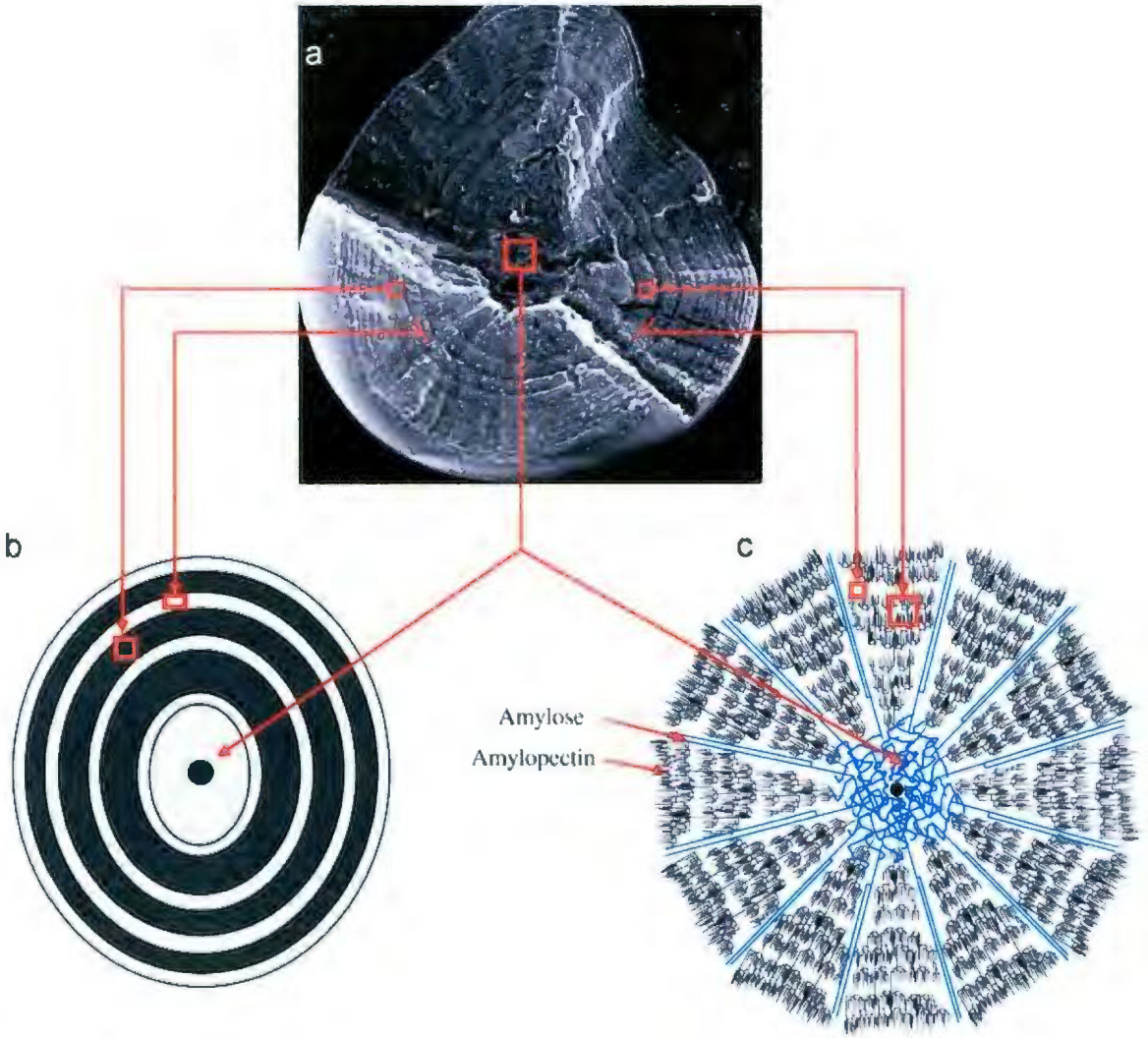
The color of the amylose-iodine complex can vary according to chain length. In this case, the colors of the complexes can change from brown (DP 21-24) to red (DP 25-29), red-violet (DP 30-38), violet-blue (DP 39-46), and blue (>47), with amylose chains having DP<20 being colorless (Johns et al., 1983). It is believed that triiodide ion (I^{3-}) is the preferred form of iodine for the formation of the lipid-iodine complex and its characteristic blue color (John, Schmidt and Kneifel, 1983). Studies have also shown that the principle chromophore of the complex is the pentaiodide ion (I^{5-}) (Teitelbaum, Ruby and Marks, 1980). In any case, the pentaiodide ion can break down to form the triiodide ion and molecular ion under certain conditions, especially at elevated temperatures (Teitelbaum, Ruby and Marks, 1980).

2.3.1.1.4 Location of amylose

There still remains some controversy over the location of amylose in the starch granule. Studies on maize and potato starch suggest that amylose is heterogeneously dispersed within the starch granule among the amylopectin molecules in both the amorphous and crystalline regions rather than located in exclusive bundles (Jane et al., 1992; Jane and Shen, 1993). Jenkins and Donald (1995) demonstrated that increasing amylose content increased the size of the crystalline portion of the amylopectin cluster and that amylose acts to disrupt the packing of the amylopectin double helices within the crystalline lamellae. Others suggest that amylose is mainly present in the amorphous growth rings, with only small amounts associated with the semi-crystalline growth ring (Montgomery and Senti, 1958). Atkin et al. (1999) suggested that the location of amylose within the granule is dependent on amylose content. That is, in starches with low amylose contents, the amylose is mainly localized in the amorphous growth rings alternating with the semi-crystalline growth rings, whereas in high amylose content starches, amylose is located in an independent region between the amylopectin center and outer surface. Furthermore, in pea starch (**Figure 2-8**), the amorphous core of the starch granule is composed mainly of amylose, while the outer part of the granule is predominantly amylopectin interspersed with some amylose molecules (Wang et al., 2012).

Figure 2-8: Model of starch granule organization, showing the location of amylose

Adapted from Wang et al. (2012) with permission from Elsevier


a
b
c
Amylose
Amylopectin

2.3.1.2 Amylopectin

Amylopectin is one of the largest biomolecules found in nature (Falk et al., 1996). It is the major component of starch, composed of a highly branched structure (**Figure 2-5B**) built from predominantly linear chains of α-(1→4)-D-glucose residues (about 95%) connected by α-(1→6)-linkages (4-5%, Biliaderis, 1998). These branch points allow the glucose molecules within amylopectin to form a highly branched and compact structure. Because of their branched nature, amylopectins have relatively low intrinsic viscosities (120-122 mL/g), despite their molecular weight (Biliaderis, 1998).

Amylopectin is larger in comparison to amylose, having an average molecular weight in the magnitude of 10^7-10^9 (Aberle, 1994; Biliaderis, 1998; Morrison and Karkallas, 1990). The degree of polymerization of amylopectin on average ranges from 0.7 to 26.5 x 10^3 (Pérez and Bertoft, 2010). The molecular size, shape, structure and polydispersity of the amylopectin molecule vary with biological origin.

2.3.1.2.1 Amylopectin structure

The structure of amylopectin is generally considered to be a cluster model, producing alternating amorphous and crystalline layers in the starch granule (Robin et al., 1974). The starch structure model proposes that the crystalline lamellae are made up of double helical amylopectin side chain clusters, which are inter-leaved with amorphous lamellae of the amylopectin branching regions (Hizukuri, 1986; Hoover and Zhou, 2003). According to the cluster model, the structural periodicity of semicrystalline starch granules is formed by repeating layers, which are approximately 9-10 nm thick and

consist of crystalline lamellae with 5-6 nm thickness and amorphous lamellae of about 4-5 nm thickness (Robin et al., 1974). It has also been suggested that some amylose chains co-crystallize with the short-chain amylopectins within the crystalline lamellae or form amylose tie chains (Jenkins & Donald, 1995; Zobel, 1988).

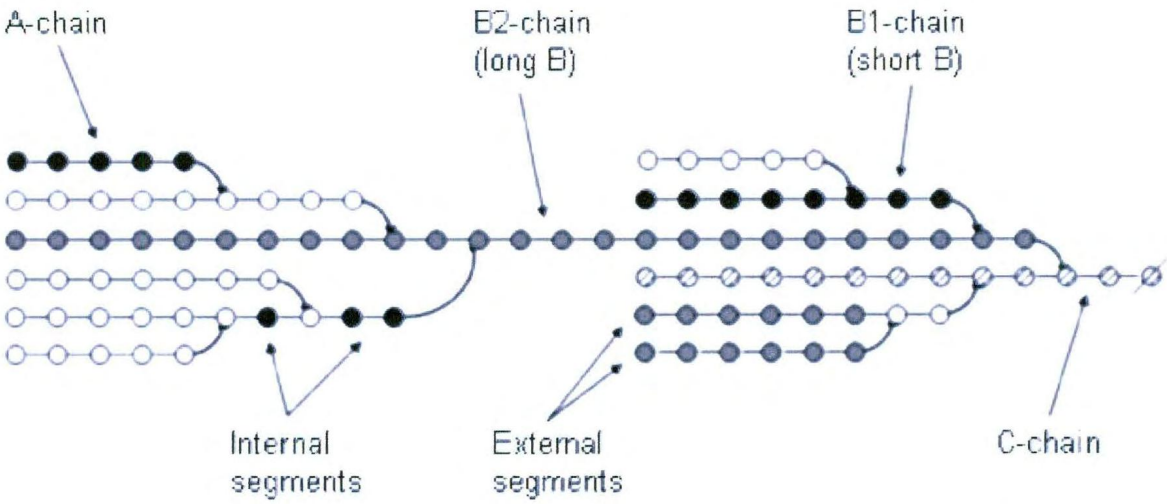
2.3.1.2.2 Amylopectin chain-length distribution

Amylopectin structure has been determined using specific α-D-(1→6) glucosidases, known as debranching enzymes (mainly isoamylase and pullulanase). Amylopectin chains are generally much shorter than amylose, with chain lengths averaging 18-25 glucose units long (Hizukuri, 1985; Biliaderis, 1998).

The amylopectin macromolecule is composed of several linked chains (**Figure 2-9**), classified according to their lengths and position within the starch granules (Hizukuri, 1985). The A-chains (DP 12-16) are linked to the macromolecule only by the reducing end group, whereas B-chains are similarly linked but also carry one or more A-chains. The C-chains carries the only reducing group in the amylopectin molecule (Peat, Whelan and Thomas, 1952). The A:B chain ratio is an important parameter in considering the brancing characteristics. Hizukuri (1986) showed the presence of different lengths of B-chains: B_1 chains which carry only one cluster and B_2, B_3 and B_4 chains which link through two, three and four clusters, respectively. The average chain lengths of B_1, B_2, B_3 and B_4 range from 20-24, 42-48, 69-75, and 101-109, respectively (Gernat et al., 1993; Hizukuri, 1986; Wang and White, 1994).

Figure 2-9: Structure of amylopectin branch chains whereby circles denote glucosyl residues, horizontal lines (1→4) linkages, and bent arrows (1→6) linkages

Adapted from Pérez and Bertoft (2010) with permission from John Wiley & Sons


A-chain
B2-chain
(long B)
B1-chain
(short B)
Internal
segments
External
segments
C-chain

The most exterior chains (A and B_1) form double helices within the native starch granules and are packed into the lamellae crystallites, while the inner chains (B_2, B_3, and B_4), act as connecting chains in the amylopectin molecule (Hoover et al., 2010). The molar ratio of short to long chains varies between 3:1 and 12:1, depending on the starch origin (Hizukuri, 1985).

The amylopectin chain length varies depending on the starch source. Generally, cereal starches have shorter glucose chains in both the long and short chain fractions and larger amounts of short chain fractions, compared to tuber and root starches (Hizukuri, 1985). **Table 2-2** summarizes the amylopectin chain length distribution of pulse starches, whereby all starches contained the greatest amounts of medium length chains (DP 13-24), followed by DP 6-12 and DP 25-26.

The polymodal chain distribution in amylopectin is consistent with the cluster method discussed previously (Robin et al., 1974). Conformational analysis and molecular modeling of the branching point of amylopectin revealed that the side chains can remain parallel to the main backbone strand, allowing formation of double helices and the development of dense three-dimensional structures during deposition of the growing polymer in the amyloplast (Biliaderis, 1998).

Table 2-2: Amylopectin chain length distribution of selected pulse starches

Starch Source	Average Chain Length	Distribution (%)				Reference
		DP 6-12	DP 13-24	DP 25-36	DP 37-50	
Chickpea	16.9-17.2	28.7-30.6	54.8-56.0	14.6-15.6	-	Chung *et al.*, 2008a
Adzuki Bean	21	-	-	-	-	Yoshimoto et al., 2001
Field pea	22.9-24.2	16.2-19.6	48.2-52.9	13.9-17.5	16.2-19.4	Ratnayake *et al.*, 2001
Grass pea	19.2-19.3	18.8-19.1	59.2	16.8-17.5	4.5-4.8	Jayakody et al., 2007
Kidney bean	17.5-17.8	23.7-25.1	59.3-59.7	15.6-16.6		Chung et al., 2008c
Lentil	17.3-17.4	26.0-26.9	57.8-58.4	15.3-15.6	-	Chung *et al.*, 2008a
Navy bean	17.7	24.3	59.8	16.0		Chung et al., 2008c
Yellow pea	17.4-17.6	24.1-25.4	59.1-59.9	15.4-16.0	-	Chung *et al.*, 2008a

2.3.1.2.3 Crystallinity and polymorphic pattern

X-ray diffraction techniques have been used to measure the degree of crystallinity of a starch granule. As with amylose, amylopectin molecules also arrange themselves to form double helices. As previously mentioned, these helices are formed by the outer unbranched A-chains and the shortest inter-branched B-chains (B_1). This formation of the double helices can either occur between branch chains from two amylopectin clusters, or among branch chains within the same cluster (Gallant, Bouchet and Baldwin, 1997).

Studies have shown that the crystallinity of starches increase with increased double helix content, thereby suggesting that the helices are packed into the crystalline structure (Lopez-Rubio et al., 2008). These helices can pack into either an A-type or B-type polymorphic pattern, depending on several factors. The lateral distance between the helical A and B amylose forms are almost identical, suggesting an interconversion between the two structures (Sarko and Wu, 1978). B-type amylopectin branching are clusters, thus forming the smaller amorphous lamellae, whereas the A-type are scattered into the amorphous and crystalline regions (Jane et al., 1997).

Native starch molecules can be characterized by A-type (cereal starches), B-type (tuber and root starches and high amylose starches) or C-type X-ray diffraction patterns (Buléon et al., 1998; McPherson and Jane, 1999; Lawal and Adebowale, 2005; Singh, Sandhu and Kaur, 2004). According to their X-ray patterns (**Figure 2-10**) A-type starches exhibit reflections at approximately 15.3, 17.0, 18.0, 20.0, and 23.4° 2θ angles, B-type starches at approximately 5.5, 15.0, 17.0,19.7, 22.2, and 24° 2θ angles, and C-type

Figure 2-10: X-ray diffraction patterns of A-, B-, and C-type starches

Adapted from Ratnayake and Jackson (2008) with permission from Elsevier

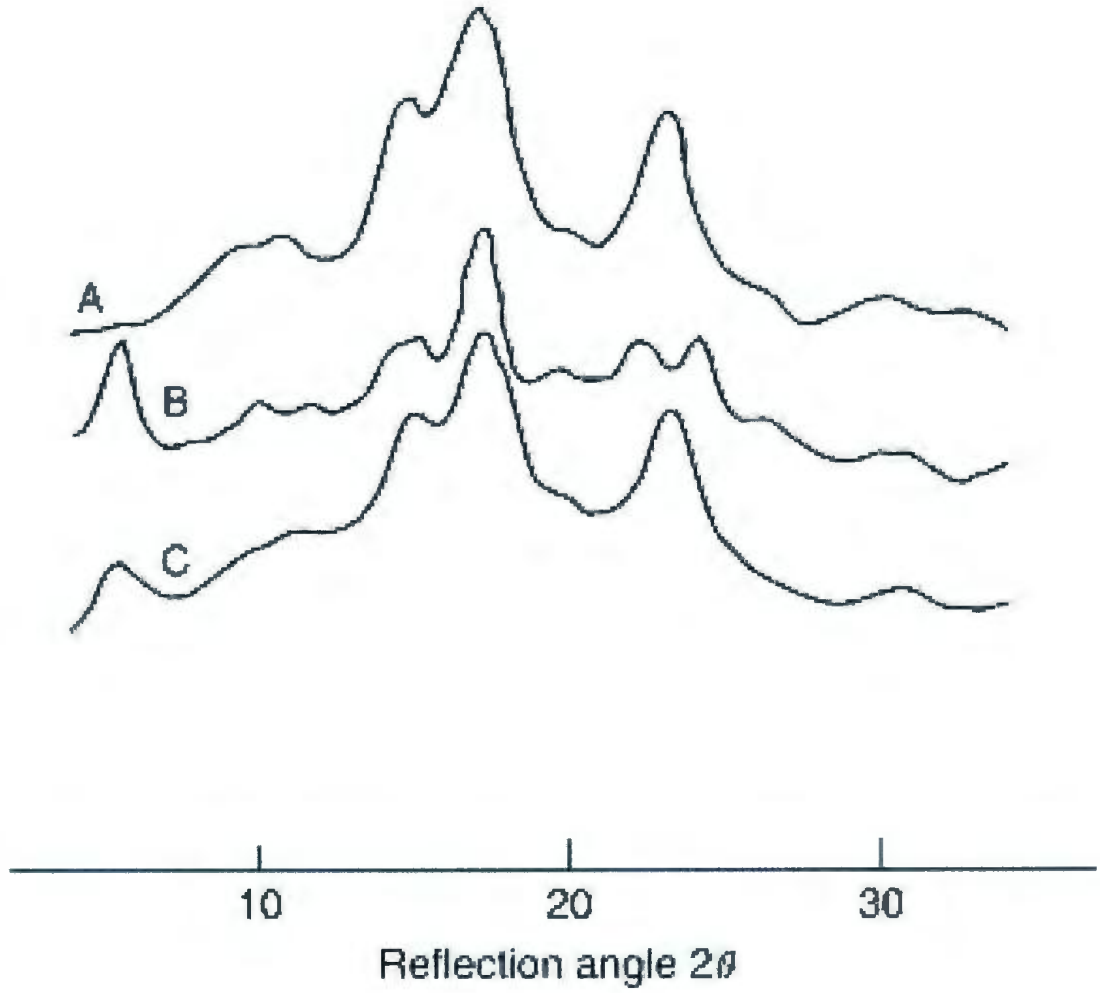

A
B
C
10
20
30
Reflection angle 2ø

exhibit major peaks at approximately 5.5, 17.0, 18.0, 20.0, and 23.52° 2θ (Jayakody et al., 2005; Jayakody et al., 2007; Zobel 1998).

Most pulse starches exhibit a 'C'-type diffraction pattern, which is an intermediate between the A-type and B-type. In the C-type starches, the B polymorphs are arranged centrally while the A polymorphs are located peripherally within the granules (Bogracheva et al., 1998). Polymorphic transition by thermal treatment has been observed to follow the order B-, C-, and A-types (Jacobs and Delcour, 1998), whereby the A-type is more thermodynamically stable and cannot be converted to the B-type or C-type by hydrothermal treatment (Kiseleva et al., 2004).

2.3.1.2.4 Degree of crystallinity

X-ray crystallinity includes determination of the absolute crystallinity, the difference between the amorphous and crystalline component of the X-ray diffractogram, and relative crystallinity, which can be defined as the percentage of crystalline regions with respect to the total material (Buélon et al., 1998). Relative crystallinity is commonly in the range of 15-45% for starch granules (Lopez-Rubio et al., 2008).

Several factors can affect the crystallinity of starch granules including: sample preparation method (Cottrell et al.., 1995), moisture content (Jayakody et al., 2005; Buélon et al., 1998), amylopectin chain length (Biliaderis et al.,1981), degree of amylopectin branching (Cottrell, 1995), crystallite number (Jayakody et al., 2005), double helical orientation (Jayakody et al., 2005), extent of double helical packing within the crystalline

lamellae (Jayakody et al., 2005), extent of amylopectin crystallite disruption by amylose (Donald and Jenkins, 1995), and amylose content (Waduge et al., 2006).

Table 2-3 summarizes crystallinity differences among pulse starches, demonstrating that all pulse starches exhibit the characteristic C-type crystalline pattern except for the B-type crystalline pattern of wrinkled pea. Relative crystallinity of all reported pulse starches were in the range of 17 to 34%.

2.3.1.2.5 Semi-crystalline structure of the starch granule

Various techniques have been used to study the structure of the starch granule, including electron microscopy, wide angle X-ray scattering and diffraction (WAXD), small angle X-ray scattering (SAXS), ^{13}C-NMR, viscometry and differential scanning calorimetry (DSC) (Biliaderis, 1998).

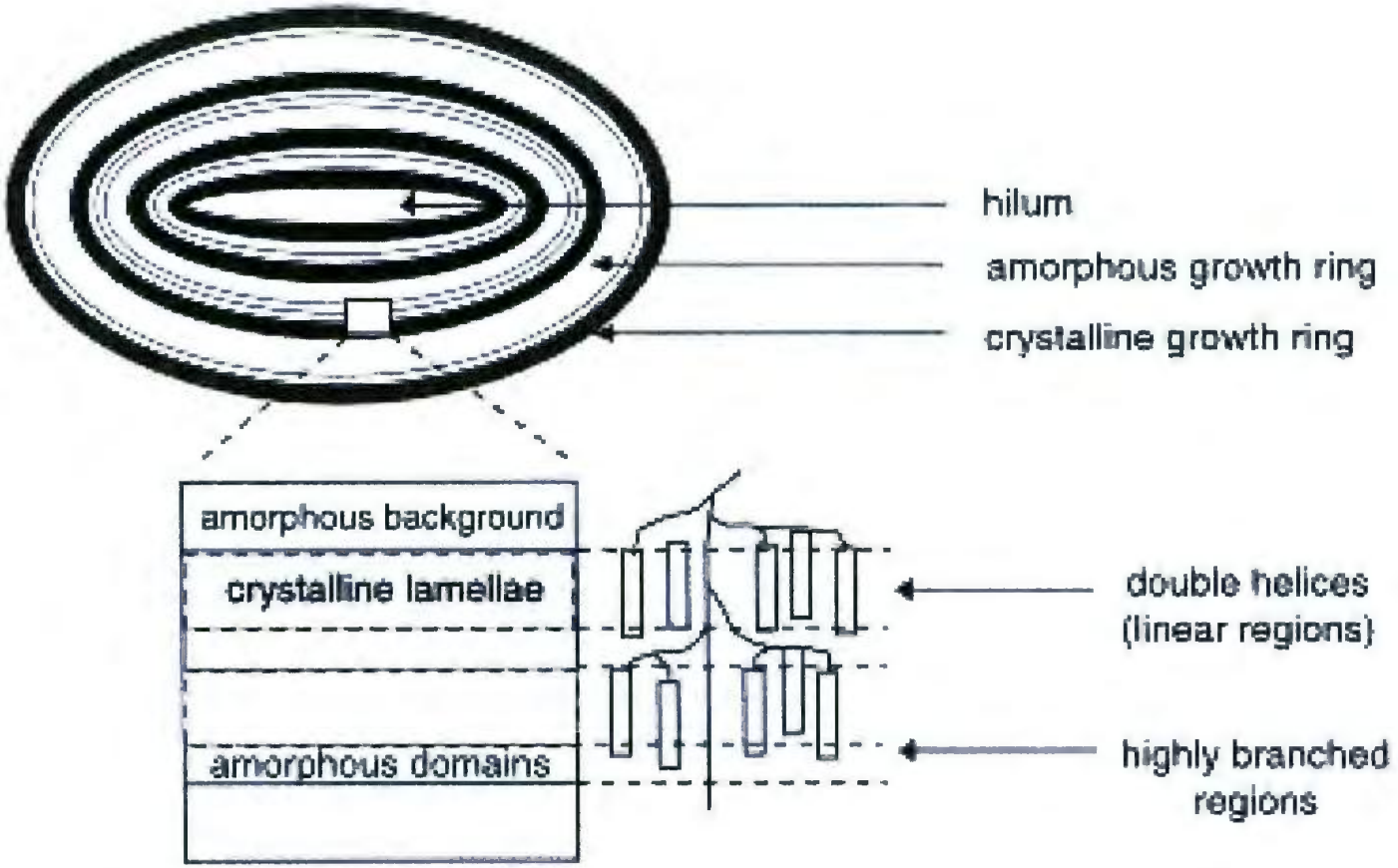
The proposed structure for a starch granule is that it consists of alternating concentric growth rings of amorphous and crystalline regions as shown in **Figure 2-11**. The crystalline regions are formed by double helices of amylopectin side chains packed laterally into a lattice, while the amorphous region is characterized by both amylose and amylopectin branching points (Jenkins et al., 1994; Jane, Wong and McPherson, 1997), whereby the longer amylopectin chains are thought to pass from the crystalline region into the amorphous region of the lamellae (Qui et al., 2004; Yamaguchi et al., 1979). A and B-type starches also differ in the thickness of their lamellae with A and B-type starches having a combined thickness of amorphous plus crystalline regions of 9 and 9.2nm , respectively (Jane, 1997). The amorphous region accounting for approximately 70% of the

Table 2-3: Crystallinity, crystalline pattern, and polymorphic composition of pulse starches

Starch Source	Crystallinity (%)	Crystalline Pattern	"B" Polymorph Content (%)	Reference
Chickpea	17.6-18.0	C	-	Hoover & Ratnayake, 2002
	26-27.6	C	-	Huang et al, 2007, Sandhu & Lim, 2008
	21.2-27.4	C	-	Xu et al., 2013
Beach Pea	-	C	-	Chavan et al., 1999
Black Bean	17.0-21.7	C	-	Hoover & Ratnayake, 2002
	32.7	C	33.1	Zhou et al., 2004
Black Gram	28.1	C	-	Sandhu & Lim, 2008
Cowpea	26	C	-	Huang et al, 2007
Field Pea	20.4-25.1	C	22.1-25.6	Ratnayake et al., 2001
	27.6	C	-	Sandhu & Lim, 2008
Grass Pea	33-34	C	-	Chavan et al., 1999, Jayakody et al., 2007
Lentil	18.7	C	-	Hoover & Ratnayake, 2002
	30.6-31.7	C	28.1	Zhou et al., 2004, Sandhu & Lim, 2008
Mung Bean	29.1	C	-	Hoover et al., 1997, Sandhu & Lim, 2008
Navy Bean	19.5-20.5	C	-	Hoover & Ratnayake, 2002
Pigeon Pea	33.4	C	-	Hoover et al., 1993, Sandhu & Lim, 2008
Pinto Bean	25.0-25.5	C	-	Hoover & Ratnayake, 2002
	33.4	C	32.1	Zhou et al., 2004
Smooth Pea	19.9-20.3	C	-	Hoover & Ratnayake, 2002
	30.3	C	28.1	Zhou et al., 2004
Wrinkled Pea	17.7	B	92.2	Zhou et al., 2004
Yellow Pea	21	C	-	Huang et al, 2007

Figure 2-11: Semi-crystalline structure of a starch granule

Adapted from van der Burgt et al. (1999) with permission from Elsevier


hilum
amorphous growth ring
crystalline growth ring
amorphous background
crystalline lamellae
amorphous domains
double helices
(linear regions)
highly branched
regions

starch granule consists of free amylose, lipid-complexed amylose, and some branched amylopectin, (Hizukuri, 1986; Oostergetel and Van Bruggen, 1993).

2.3.2 Minor Components

Starch also contains smaller quantities of lipids, proteins, and trace amounts of minerals. Although they are present in comparatively small amounts, they can greatly influence starch properties including digestibility, swelling, retrogradation, amylose leaching, pasting properties and granule integrity (Han and Hamaker, 2002; Baldwin, 2001; Appleqvist and Debet, 1997).

2.3.2.1 Lipids

Lipids associated with starch granules can be present both on the surface and inside the starch granule, as well as non-starch lipids such as those present in the aleurone layers and germ of the starch grain (Morrison, 1981; 1988). Surface lipids are mainly triacylglycerols, but also smaller quantities of free fatty acids, glycolipids, and phospholipids may be present (Morrison, 1981; Vasanthan and Hoover, 1992). Lipids within the starch granule are generally smaller, so therefore monoacylglycerols, such as lysophospholipids and free fatty acids are most abundant (Morrison 1981; Vasanthan and Hoover, 1992).

Starch lipids can also exist in free form, or otherwise bound to starch components. Free lipids are readily extracted using a chloroform/methanol solution at room temperature (Morrison 1981; Vasanthan and Hoover, 1992). Bound lipids are commonly linked to the hydroxyl groups of amylose or amylopectin via ionic or hydrogen bonds, or otherwise form amylose-lipid complexes where the lipid is located inside the hydrophobic center of the amylose double helix (Morrison, 1981) (see section 2.3.1.1.2). The extraction of bound lipids requires a hot aqueous solvent such as n-propanol/ water and long refluxing times, or acid hydrolysis to disrupt the starch granule, thereby releasing the bound lipids (Morrison 1981; Vasanthan and Hoover, 1992).

The lipid content of various pulse starches are presented in **Table 2-1**. According to this table, the amount of lipid in pulse starches in minimal, since all cultivars have less than 0.8% total lipid, with many varieties having a significantly lesser amount. **Table 2-1** also compares the relative amounts of surface and bound lipids, demonstrating that all pulse starches have significantly higher amounts of bound than surface lipids.

2.3.2.2 Proteins

The amount of protein (<3g/kg) within a starch granule varies with different species and cultivars. Proteins associated with starch granules can be classified (Baldwin, 2001) as surface proteins which have low molecular weights (5-60 kDa), or internal proteins, with higher molecular weights (60-150 kDa). Surface proteins are deposited as aggregates on the granule surface and can be readily extracted using a diluted sodium

chloride, aqueous alkali, or sodium dodecyl sulfate solutions at room temperature, whereas internal proteins are interspersed in the matrix and can only be extracted after gelatinization (Seguchi and Yamada, 1989; Mu-Forster and Wasserman, 1998). Most internal proteins are starch synthases and their isoforms (isozymes) are involved in starch biosynthesis, and thus become entrapped within the granule structure during granule synthesis (Baldwin, 2001). Starch-bound proteins influence starch digestibility, swelling, gelatinization and granular integrity (Gaillard and Bowler, 1987).

Table 2-4 outlines the nitrogen contents of several pulse starches. Note that the amount of nitrogen is relatively low in all pulse starches, indicating that the starches contain only minute amounts of protein.

2.3.2.3 Phosphorous and minerals

Starch also contains minor amounts of phosphorous and other minerals, such as calcium, potassium, magnesium, and zinc. The phosphorous present can be either in the form of lysophospholipids, phosphate monoesters or inorganic phosphates. The form and proportion of phosphorous varies depending on the type of starch. Cereal starches have mainly lysophospholipids, whereas waxy and tuber and root starches have more phosphate monoesters. Pulse starches have relatively low phosphorous contents, which are mainly in the form of phosphate monoesters.

Table 2-4: Composition of selected pulse starches

Starch Source	Yield (%)	Moisture (%)	Nitrogen (%)	Starch Damage
Chickpea[1,d,h,e]	30.4-46.3	7.4-10.9	0.09-0.10	1.6-2.1
Beach Pea[c]	12.3	10.57	0.08	4.9
Black Bean[e,k]	16.4-22.2	10.8-11.0	0.03-0.07	0.27-2.0
Cowpea[h]	-	11.5	-	-
Field Pea[j]	32.7-33.7	9.2-13.3	0.04-0.07	1.73-2.55
Grass Pea[i,c]	21.1-26.0	10.87	0.03-0.11	1.7
Green Pea[c]	30.0	10.60	0.09	1.9
Jack Bean[a,b]	-	10.16	-	-
Lentil[d,e,k]	27.4-47.1	8.6-9.87	0.04-0.06	0.15-1.6
Lima Bean[a]	-	10.16	-	-
Mung Bean[f]	31.1	10.03	0.05	-
Navy Bean[e,k]	23.8-24.9	-	0.07-0.08	1.5-1.8
Pigeon Pea[g]	29.7	10.9	0.02	2.0
Pinto Bean[e,k]	25.0-30.1	11.38-12.22	0.06-0.08	0.22-1.6
Smooth Pea[e,k]	19.4-35.8	9.47-10.47	0.02-0.08	0.4-2.0
Velvet Bean[b]	-	-	0.098	-
Wrinkled Pea[k]	21.6	11.76	0.03	3.54
Yellow Pea[h]	-	11.3	-	-

[1]Letter superscript indicates the reference: [a]Betancur-Ancona et al., 2001, [b]Betancur-Ancona et al., 2002, [c]Chavan et al., 1999, [d]Chung et al., 2008, [e]Hoover & Ratnayake, 2002, [f]Hoover et al., 1997, [g]Hoover et al., 1993, [h]Huang et al., 2007, [i]Jayakody et al., 2007, [j]Ratnayake et al., 2001, [k]Zhou et al., 2004.

2.4 Granular Morphology

Starches vary in size and shape depending on the biological origin (Jane et al., 1994), biochemistry and physiology of the plant (Badenhuizen, 1969), and age of the starch (French, 1984). Although there can be much variation in granule morphology, the shape of starch granules are usually consistent within the starch subtypes. For example, studies have shown tuber starches to be generally large, elliptical or spherical, cereal starches are small and polyhedral, and pulse starches kidney or ovoid shaped (Tester and Karkalas, 2002).

The shapes of various pulse starches are summarized in **Table 2-5** showing shapes of mostly round-oval, although kidney, elliptical, spherical, and irregular shapes are also reported. For the most part, pulse starches have simple shapes, except for wrinkled pea, which has a compound shape.

The size of starch granules can vary immensely, in which average diameters of starch granules can range from less than 1μm to more than 100 μm (Hoover, 2001). According to **Table 2-5**, pulse starches are relatively small, ranging from 6 to 26 μm.

2.5 Physicochemical Properties

2.5.1 Granular Swelling

Granular swelling provides evidence of the strength of interaction between starch chains within the amorphous and crystalline domains (Ratnayake, Hoover and Warkentin, 2002),

Table 2-5: Granule morphology and size of pulse starches

Starch Source	Granule Shape	Mean Length (μm)	Length Range (μm)	Mean Width (μm)	Width Range (μm)	Unspecified Mean (μm)	Unspecified Range (μm)
Chickpea[1,d,j,k]	oval, spherical	22-22.4	14-31	18.5-18.85	9-30	17.8-21.6	-
Beach Pea[c]	round-elliptical	17	-	11	-	-	6-17
Black Bean[d,l]	round-oval	21-22	10-41	18.8-19.4	10-37.5	-	
Cowpea[h]	oval-spherical	-	-	-	-	-	3-64
Field Pea[i]	round-elliptical	25	-	10	-	5-7	
Grass Pea[c,g]	oval-round-elliptical	21	10-60	13	-	-	13-32
Green Pea[c]	round-elliptical	35	-	22	-	-	14-33
Jack Bean[a,b]	Oval	-	-	-	-	31.5	15-60
Lentil[d,k]	round-oval-irregular	19.0-19.5	6-37	17.8-18.1	6-28	-	-
Lima Bean[a]	Oval	-	-	-	-	17.9	10-52
Mung Bean[e]	oval-round-bean shaped	-	-	-	-	-	7.1-26.1
Navy Bean[d,l]		22.5-22.8	14-28	19.1-19.2	8-32	-	-
Pigeon Pea[f]	oval-elliptical-irregular	-	-	-	-	-	8-32
Pinto Bean[d,l]	round-oval	22.0-22.5	10-42	19.0-19.2	6-32	-	-
Smooth Pea[d,l]	round-oval-irregular	22.6-23.0	8-50	20.5-21.0	8-34	-	-
Velvet Bean[b]	Oval	-		-	-	23.6	15-60
Wrinkled Pea[l]	irregular-compound	-	5-37	-	5-34	-	-

[l]Letter superscript indicates the reference: [a]Betancur-Ancona et al., 2002a, [b]Betancur-Ancona et al., 2002b, [c]Chavan et. al., 1999, [d]Hoover & Ratnayake, 2002, [e]Hoover et al., 1997, [f]Hoover et al., 1993, [g]Jayakody et al., 2007, [h]Okechukwu & Rao, 1996, [i]Ratnayake et al., 2001, [j]Singh et al, 2004, [k] Xu et al., 2013,[l]Zhou et al., 2004

and was hypothesized by Tester and Morrison (1990a) to be primarily a property of amylopectin, with amylose acting as a diluent. The heating of starch in excess water results in the disruption of its crystalline structure (due the dissociation of hydrogen bonds), resulting in the linkage of water molecules to the exposed hydroxyl groups of the starch components via hydrogen bonding, which leads to increased granular swelling and amylose leaching (Liu, Ramsden and Cork, 1999; Tester and Morrison, 1990a,b).

The extent of granular swelling can be reported as either swelling power (SP) or swelling factor (SF) and can be measured by gravimetry (Leach, McCowden and Schoch, 1959), colorimetry (Tester and Morrison, 1990a), or by laser light scattering (Ziegler, Thompson and Cassasnovas, 1993). SP is defined as the weight of a sedimented starch gel, relative to its dry weight, determined after gelatinizing the starch in excess water at a given temperature and time, followed by centrifugation (Crosbie, 1991). SP, on a weight basis (g/g), measures both the intergranular and intragranular water (Crosbie, 1991). SF is defined as the ratio of the swollen volume to the initial volume of air dried starch and hence has no units (Tester and Morrison, 1990 a,b). The measurement of SF is based on the observation that blue dextran dye (molecular weight 2x106 Da) can dissolve in the supernatant and interstitial water but not in the intragranular water. Thus, SF only measures the water that enters the granule and contributes to volume expansion upon heating (Tester and Morrison, 1990a,b).

The pattern of granular swelling varies according to starch type. That is, starches from pulses, roots and tubers generally exhibit a single-stage swelling patterns (Hoover and Sosulski, 1986; Hoover, 2001), whereas cereal starches normally display two-stage

swelling (Leach, McCowden and Schoch, 1959). Single stage swelling patterns indicates relaxation of bonding forces within starch granules over one temperature and not over multiple temperatures because bonding forces are more uniform and stronger, whereas, two-stage swelling indicates that there are two types of forces within the granule which require different energy inputs to weaken starch chain interactions (Hoover and Sosulski, 1991; Soni and Agarwal, 1983).

Granular swelling of several pulse starches are listed in **Table 2-6**. Unfortunately, differences in measuring granular swelling (swelling power vs swelling factor), makes comparisons among pulse starches difficult. Most pulse starches, exhibit no measurable granular swelling at temperatures below 60°C, but show pronounced swelling at temperatures above 70°C. This was attributed to the high amylose content of pulse starches. In high amylose starches, amylose chains are closely packed within the amorphous domains of the granule, resulting in strong interactions (via hydrogen bonds) between adjacent chains. Consequently, a high input of thermal energy would be required to disrupt the above interactions.

2.5.1.1 Factors affecting granular swelling

Granular swelling is also influenced by an interplay of several factors including botanical source (Debet and Gidley, 2006), growth temperature (Myllärinen et al., 1998), amylose content (Sasaki and Matsuki, 1998; Tester and Morrison, 1990 a,b; Waduge et al., 2006), lipid-amylose complexes (Tester and Morrison, 1990 a,b; Waduge et al., 2006, Hoover

Table 2-6: Granular swelling and amylose leaching of pulse starches at 60-90°C

Starch Source	Parameter	Granular swelling				Amylose leaching (%)			
		60°C	70°C	80°C	90°C	60°C	70°C	80°C	90°C
Chickpea	SP [1,2h]	4.6-5.3	4.6-5.3	8.4-9.4	9.2-10.4	1.2-2.1	3.3-4.1	4.5-5.0	5.5-5.9
	SF [f]	15.0-18.2	-[3]	-	-	18.5-21.7	-	-	-
	SF [d]	5.1-9.4	13.3-16.1	19.1-23.1	22.6-23.4	4.0-5.1	11.9-13.0	15.2-16.0	17.2-19.1
Beach Pea	SF[e]	8.55	16.73	18.43	24.92	-	3.43	7.54	11.55
Black Bean	SF[f]	24.5-29.6	-	-	-	24.0-29.1	-	-	-
	SF [i]	-	-	8.2-17.7	-	-	-	13.6-16.5	
Grass Pea	SF[e]	1.56	10.02	13.03	19.58	-	6.25	15.07	17.68
Green Pea	SF[e]	8.94	17.70	21.11	28.01	-	6.16	14.33	16.69
Field Pea	SF[i]	8.4-8.6	13.3-13.8	19.2-19.4	26.4-26.5	10.1-10.7	15.1-16.6	18.1-20.3	25.1-26.6
Lentil	SP[h]	4.3-4.4	7.4-7.8	9.7-10.5	10.9-11.9	0.9-1.0	5.1-5.2	7.4-7.6	8.5-8.8
	SF [f]	21.0-21.5	-	-	-	24.5-24.8	-	-	-
	SF[j]	-	-	16.0-18.4	-	-	-	13.6-17.7	-
	SF [d]	3.7-5.0	14.0-15.4	22.2-23.7	22.5-24.5	2.2-2.3	15.8-16.2	20.7-20.8	22.5-22.6
Jack Bean	SF[b]	-	-	-	33.9	-	-	-	
Mung Bean	SF[g]	3.9	16.7	31.9	37.8	3.6	26.7	32.3	35.1
Kidney Bean	SF[e]	5.0-5.6	11.2-11.4	20.2-21.6	20.7-24.1	1.4-1.5	9.5	18.9-20.4	22.7-24.8
Navy Bean	SF[f]	12.0-12.5	-	-	-	11.8-12.1	-	-	-
	SF [e]	5.0	10.9	23.3	24.4	1.0	8.3	18.5	21.9
Pinto Bean	SF[f]	10.0-10.5	-	-	-	11.8-12.2	-	-	-
	SF[j]	-	-	9.9-10.4	-	-	-	11.0-13.0	-
Smooth Pea	SF[f]	16.0-18.5	-	-	-	20.0-22.7	-	-	-
	SF[j]	-	-	16.2-16.6	-	-	-	17.6-17.8	-
Velvet Bean	SP[a]	2.5	5.0	14.8	17.5	2.5	5.1	15.0	15.2
Wrinkled Pea	SF[j]	-	-	3.4	-	-	-	11.1	-
Yellow Pea	SF[h]	4.3-4.4	7.4-7.8	9.7-10.5	10.9-11.9	0.9-1.0	5.1-5.2	7.4-7.6	8.5-8.8

[1]Letter superscript indicates the reference: [a]Betancur-Ancona et al., 2002a, [b]Betancur-Ancona et al., 2002b, [c]Chavan et al., 1999, [d]Chung et al., 2008a, [e]Chung et al., 2008c, [f]Hoover & Ratnayake, 2002, [g]Hoover et al., 1997, [h]Huang et al., 2008, [i]Ratnayake et al., 2001,[j]Zhou et al., 2004.

[2]SP=Granular swelling measured as swelling power (g/g), SF=Granular swelling measured as swelling factor (%)

[3] – indicates not reported in study

and Manual, 1995), amylopectin structure (Sasaki and Matsuki, 1998; Tester, Morrison, and Schulman, 1993), amylopectin chain length distribution (Srichuwong et al., 2005), granule size (Vasanthan and Bhatty, 1996), granule integrity (Sandhya, Rani and Bhattacharya, 1989), crystallinity (Jayakody and Hoover, 2002), phosphorous content, (Singh et al., 2003; Gunaratne and Hoover, 2002), pH (Lawal and Adebowale, 2005), extent of starch damage (Tester, Debon and Karkalas, 1998), extent of interaction between starch chains within the amorphous and crystalline domains of the granule (Waduge et al., 2006; Tester; Debon and Sommerville, 2000; Zhou, Hoover and Liu, 2004), chemical modifications (Landerito and Wang, 2005), and surface proteins and lipids (Debet and Gidley, 2006).

Granular swelling is accompanied by leaching of granular constituents, predominantly amylose, into the external matrix which results in a dispersion of swollen granules in a continuous matrix (Singh, Sandhu and Kaur, 2004). Increased crystallinity increases granular stability, thereby reducing the extent of granular swelling (Hoover and Ratnayake, 2002).

2.5.2 Amylose Leaching (AML)

Amylose leaching (**Table 2-6**) occurs when starch granules are heated in the presence of water and provides information on the interactions between amylose-amylose chains and/or amylose-amylopectin interactions in the interior of the starch granule. Amylose leaching is especially important in the food industry where it is essential for gel

formation, yet causes unwanted stickiness in pasta and potato flakes (Hoover and Hadziyev, 1981).

The extent of amylose leaching has been shown (Hoover et al., 2010) to increase progressively with increase in temperature. As with granular swelling, amylose leaching is not observed in pulse starches at temperatures below 60°C, which is likely due to the strong interactions between amylose molecules (Hoover et al., 2010).

2.5.2.1 Factors affecting amylose leaching

Amylose leaching is affected by numerous factors including temperature (Hoover and Vasanthan, 1994; Gunaratne and Hoover, 2002), total amylose content (Nakazawa and Wang, 2003), interactions between amylose chains and/or amylose-amylopectin chains (Waduge et al, 2006; Hoover and Vasanthan, 1994), the amount of lipid-complexed amylose (Hoover and Vasanthan, 1994; Jayakody and Hoover, 2002; Nakazawa and Wang, 2003), and granular size (Lindeboom, Chang and Tylera, 2004).

2.5.3 Gelatinization

Gelatinization is an energy absorbing process whereby the internal structure of the granule is broken down and the whole granule disintegrates releasing the polysaccharide into the surrounding medium (Kaur and Singh, 2005; 2006). More specifically, gelatinization is an irreversible change of granule swelling and melting of starch

crystallites when native starch is heated in excess water at specific temperature ranges and moisture levels (Jacobs and Delcour, 1998; Eliasson and Gudmundsson, 1996), resulting in the collapse of molecular order within the starch granule. Evidence for the loss of molecular order includes irreversible granular swelling, loss of birefringence and crystallinity, viscosity development, and solubilization of starch (Sayar, Koksel and Turhan, 2005)

During starch gelatinization, two processes occur: first, the endothermic process of the starch crystallite melting, which is followed by the exothermic process of the lipid-amylose complex formation (Eliasson and Gudmundsson, 1996). The phase change during gelatinization may be due to various phenomena, including the diffusion of water into the granules, water uptake by the amorphous background region, hydration and radial swelling of the starch granules, leaching of amylose into the solution, increased viscosity, loss of birefringence and/or crystalline order, unraveling and dissociation of double helices in the crystalline regions, and starch solubilization (Biliaderis, 1998; Jenkins et al., 1994; Hoover and Hadziyev, 1981).

2.5.3.1 Mechanism of gelatinization

It has been postulated that in excess water, gelatinization is a swelling driven process in which the water uptake in the amorphous regions is accompanied by swelling of these regions, which causes destabilization of the amylopectin crystallites within the crystalline lamellae and thus are ripped apart (Tester and Debon, 2000; Jenkins et al.,

1994). These crystallites melt cooperatively. Furthermore, this process is rapid for an individual crystallite but over a wide range for the entire starch granule. Gelatinization at the molecular level involves the uncoiling of the external amylopectin chains that are packed together in a cluster of double helices, via the disruption of the hydrogen bonds formed during coiling.

Starch gelatinization is thought to occur in two stages, the first being a slow side-by-side dissociation of helices, followed by the second stage involving the rapid helix-coil transition (Waigh et al., 2000). **Figure 2-12** outlines the sequence of gelatinization at low, intermediate, and excess water conditions.

2.5.3.2 Methods for measuring gelatinization

There is a wide array of methods used to study starch gelatinization, many of which study changes in the properties of the starch granule, which include: visco-amylography, which measures changes in viscosity due to granular swelling and solubilization, loss of granule birefringence, which determines the extent of crystallite melting or changes in crystallite orientation that occur during the initial stage of granular swelling using polarized light, and thermal analysis, which studies the melting of amylopectin disordering of double helices (Ziegler et al., 1993). Other methods include swelling factor or swelling power, measuring changes in the granular volume (Tester and Morrison, 1990a), wide angle X-ray scattering (WAXS), determines loss of granule crystallinity (Zobel at al., 1988), small angle X-ray scattering (SAXS), differentiates

Figure 2-12: Gelatinization mechanism at different starch: water ratios showing:

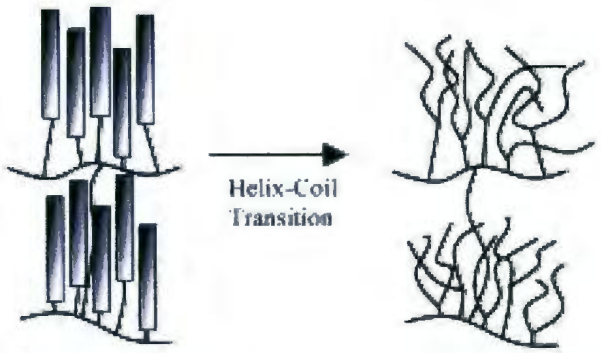
A) The single stage process in the gelatinisation of starch at low water content

B) The two stage process involved in the gelatinisation of starch in limiting water

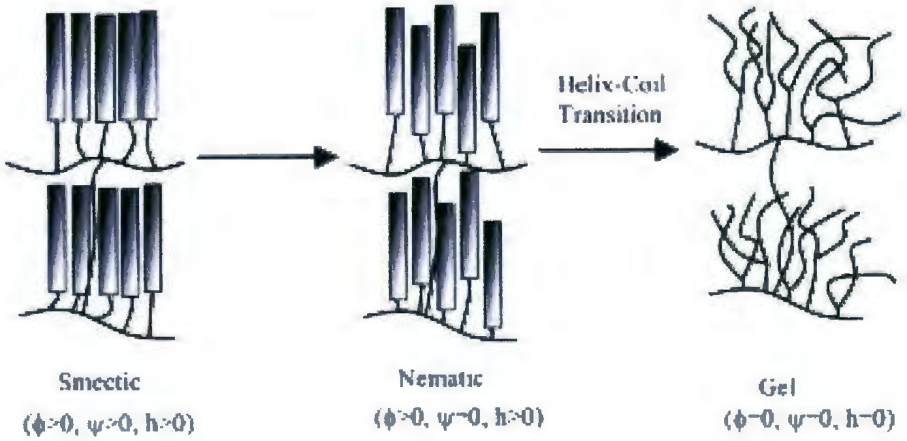
C) The two stage process involved in the gelatinisation of starch in excess water which involves a slow dissociation of the helices side by-side with an immediate helix-coil transition occurs as a secondary effect

Adapted from Waigh et al. (2000) with permission from John Wiley & Sons

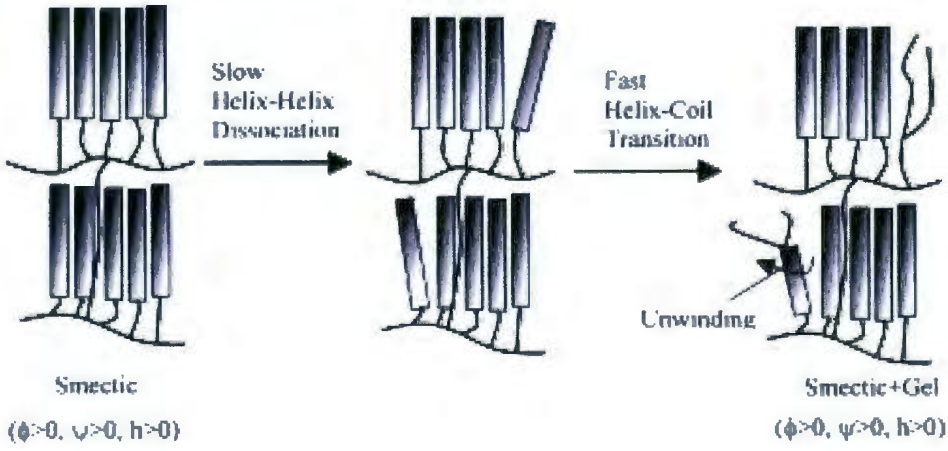
a) Low Water Content Gelatinisation



b) Intermediate Water Content Gelatinisation.



c) Excess Water Content Gelatinisation.



electron densities between amorphous and crystalline lamellae (Cameron and Donald, 1992), and confocal scanning laser microscopy (CSLM), which measures swelling of starch granule over time (Velde, Riel and Tromp, 2002).

Differential scanning calorimetry (DSC) is most widely used to measure gelatinization parameters (Tester and Karkalas, 2001). In this case a single DSC endotherm is formed from the heating of the starch in the presence of excess water and reflects the endothermic transition of the heterogeneous granule population (Karim et al., 2007). From this endotherm, the gelatinization parameters including the onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c), and gelatinization temperature range (T_c-T_o) which corresponds to the stability of crystallites, can be determined. From the endotherm, the enthalpy of gelatinization (ΔH) can be calculated as the area under the gelatinization peak (Meares et al., 2004).

These gelatinization parameters are influenced by the molecular structure of the crystalline region that corresponds to the distribution of amylopectin short chains (DP 6-11) and not by the crystalline region corresponding to the amylose/amylopectin ratio, or the overall crystallinity of amylopectin, which increases with increasing amounts of amylopectin (Gernat et al., 1993; Noda et al., 1998; Tester and Morrison, 1990a,b). In this case, low T_o, T_p, T_c, and ΔH illustrate the presence of short amylopectin chains (Noda et al., 1998). Tester and Debron (2000) have shown that a rapid drop in crystallinity occurs between the onset and the peak temperatures of the DSC endotherm, and that after the conclusion temperature, all amylopectin double helices are dissociated. The representation of ΔH in terms of structure is still somewhat controversial, with some

authors suggesting that ΔH reflects the loss of double helical order in the starch molecule (Cooke and Gidley, 1992; Zhou, Hoover and Liu, 2004), while others believe it reflects the overall crystallinity of amylopectin (Hoover et al., 2010; Tester and Debon, 2000; Tester and Morrison, 1990a,b).

2.5.3.3 Factors affecting gelatinization

Many factors affect gelatinization parameters. These include but are not limited to amylose content (Pernoni, Rocha and Franco, 2006; Hoover and Manuel, 1996), amount of lipid-complexed amylose (Morrison, 1995), lipid content (Russell, 1987; Evans, 1986), amylopectin chain length (Wang et al., 2006; Noda et al., 1998), stability of the amorphous region (Biliaderis, 1990), starch to water ratio (Farhat, Oguntona and Neale, 1999; Ziegler, Thompson and Cassanovas, 1993; Cotrell et al., 1995), starch damage (Waduge et al., 2006; Wang et al, 2006; Noda et al., 1998), double helical content (Jacobs et al., 1998), presence of solutes (sugars, phosphates, salts) (Chiotelli and Meste, 2002), crystallite size and perfection (Singh, McCarthy and Singh, 2006; Perera, Lu and Jane, 2001; Tester et al., 1991), botanical source (Peroni et al., 2006; Wong et al., 2003; Noda et al., 1998), granule size and morphology (Jayakody et al., 2005; Karlsson and Eliasson, 2003), physical modifications (Hoover et al., 1993; Tester and Debon, 2000), heating rate (Frietas et al., 2004; Ziegler, Thompson and Cassanovas, 1993) and growth temperature (Kohyama et al., 2004; Tester et al., 1999).

Table 2-7 demonstrates the gelatinization parameters of various pulse starches, which compares the T_o, T_p, T_c, and ΔH. Among pulse starches, there is a wide range of enthalpy values, ranging from 2 to 18.5. More specifically, in chickpea where several cultivars were compared, ΔH values ranged widely from 2.18 to 18.5 (J/g). There were also no obvious differences among the T_o, T_p, T_c values of the various pulse starches.

2.5.4 Pasting properties

Pasting refers to changes in the starch upon further heating after gelatinization, including further swelling and leaching of polysaccharides from the starch granules, and the eventual disruption of the starch granule, and increased viscosity which occurs with the application of shear forces (Tester and Morrison, 1990b; Atwell et al., 1988). Therefore, a paste can be defined as a viscous mass composed of both a continuous phase of amylose and/or amylopectin compounds, as well as a discontinuous phase consisting of granule fragments (Whistler and BeMiller, 1997).

The use of starch in the food, paper and textile industries is dependent on starch paste viscosity (Moorthy, 2002). Starch stability when exposed to heating and shear forces is important in the food industry for products that are subjected to high temperature processing, so that starch degradation can be limited.

Table 2-7: Gelatinization properties of pulse starches at varying starch to water ratios

Starch source	Starch:water Ratio	Gelatinization parameter T_o (°C)	T_p (°C)	T_c (°C)	Enthalpy ΔH (J/g)
Chickpea[l,d,f,l,n]	1:3	59.3-66.2	64.7-72.5	69.8-81.5	2.18-12.4
Beach Pea[c]	1:3	60	64.5	74.2	14.2
Black Bean[f,o]	1:3	61.0-66.9	69.9 – 76.5	81.2-86.7	12.1-20.1
Black Gram[l,m]	1:3	66.1-71.3	71.0-76.2	76.0-80.4	6.7-9.4
Cowpea [h]	1:3	70.5	75.4	81.0	15.2
Field Pea[k,l]	1:3	55.9-61.4	61.4-67.5	66.5-76.0	11.2-12.3
Green Pea[c]	1:3	69.4	72	76.3	6.6
Grass Pea[c,i,]	1:3	65.7-68.3	71.0-75.5	74.2-85.4	8.5-15.3
Grass Pea[j]	1:2	60.2-61.3	67.5-68.5	74.2-74.6	12.4-12.6
Kidney Bean[e]	1:3	66.7 - 66.9	73.7 - 74.5	88.5 - 89.0	14.3 - 14.8
Lentil[d,f,l,o]	1:3	57.8-68.4	66.0-76.1	71.0-82	3.0-13.3
Lima Bean[b]	1:3	75	80.16	87	-
Mung Bean[f,g,l]	1:3	58-62.2	67.4-69	72.1-83	7.9-18.5
Navy Bean[e]	1:3	65.6 – 66.8	74.3 – 75.1	84.8 – 91.0	13.2 – 15.3
Pigeon Pea[h,l]	1:3	69.3-73.9	75.5-80.1	80.6-86.9	10.7-10.9
Pinto Bean[f,o]	1:3	63.3 – 72.5	70.9 - 76.5	80.5 - 88.8	12.2 – 16.2
Smooth Pea[f,o]	1:3	60.8 - 63.9	66.9 - 70.6	73.4 - 80.1	9.9 – 13.8
Velvet Bean[a]	1:3	70	74.8	80	-
Yellow Pea[h]	1:3	58.2	65.1	70.4	16.1

[l]Letter superscript indicates the reference: [a]Betancur-Ancona et al., 2002, [b]Betancur-Ancona et al., 2001,[c]Chavan et al., 1999, [d]Chung et al., 2008a, [e]Chung et al., 2008c, [f]Hoover & Ratnayake, 2002, [g]Hoover et al., 1997, [h]Huang et al., 2007b, [i]Jayakody et al., 2007, [j]Korus et al., 2008, [k]Ratnayake et al., 2001, [l]Sandhu & Lim, 2009, [m]Singh et al., 2004b, [n]Xu et al., 2013, [o]Zhou et al., 2004.

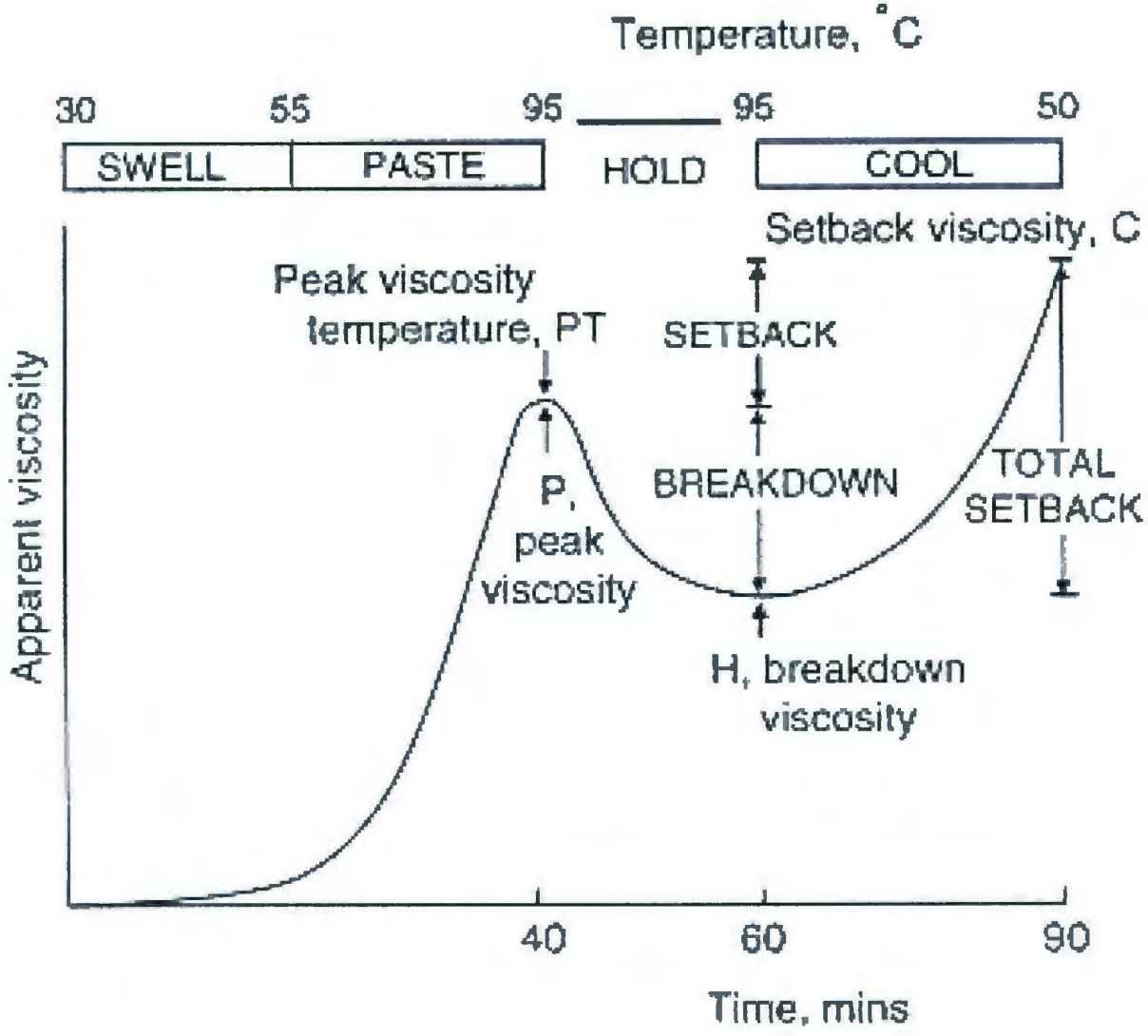
2.5.4.1 Methods for measuring pasting properties

The pasting properties of starches are commonly studied by observing changes in the viscosity of the starch system based on rheological principles (Zaidul et al., 2007). In this case, the rapid visco-analyser (RVA), Brabender visco-amylograph and rotational viscometers are extensively used for measuring starch paste viscosities. Unfortunately, Brabender visco-amylography has limited applications because it requires large amounts of starch and extended analysis time (Panozzo and McCormick, 1993). The rapid visco-analyzer, however, requires only small samples of starches, short analysis times, along with better temperature control settings. In addition, the unit of measurement for viscosity using the Brabender visco-amylograph is the Brabender unit (BU) which does not match SI units. However, viscosity measurement using the RVA is expressed as rapid visco-analyzer units (RVU) which can be expressed as standard units (1 centipose (cP)=12 RVU). Because of those advantages, rapid visco-analyzers are preferred over other methods.

Essentially, the RVA measures the viscosity of a starch suspension before, during and after gelatinization, providing information on the viscosity, shear strength, gelatinization and swelling properties of the starch (Meares et al, 2004). There is an array of information (**Figure 2-13)** that can be retrieved when a starch sample is heated and cooled in the presence of shear forces using RVA including: 1) the pasting temperature, when the initial viscosity increase is observed, 2) the peak viscosity, corresponding to the maximum starch viscosity attained during heating, 3) peak time, the time to reach peak viscosity, 4) breakdown, measured as the difference between peak and

Figure 2-13: Schematic diagram of RVA curve illustrating pasting parameters

Adapted from Karim, Norziah and Seow (2000) with permission from Elsevier


Temperature, °C
30
55
95
95
50
SWELL
PASTE
HOLD
COOL
Setback viscosity, C
Peak viscosity temperature, PT
SETBACK
BREAKDOWN
TOTAL SETBACK
P, peak viscosity
H, breakdown viscosity
Apparent viscosity
40
60
90
Time, mins

minimum viscosities during the holding cycle, 5) set-back, the difference between the minimum viscosity in the holding cycle and maximum viscosity during the cooling stage and involves retrogradation or re-ordering of the starch molecules, and 6) the final viscosity, corresponding to starch viscosity at the end of the RVA cycle (Dengate, 1984; Huang et al,, 2007).

2.5.4.2 Factors affecting pasting properties

Numerous factors influence the development of viscosity when starches are heated and cooled. Some of those factors include, but are not limited to, botanical source (Otegbayo et al., 2006; Liu, Ramsden and Cork, 1997), granule size (Singh et al., 2006; Jayakody et al., 2005), starch concentration (Jacobs et al., 1995), granule swelling (Doublier, Llamas and LeMeur, 1987), amylopectin content (Singh et al., 2006), leaching of amylose and other macromolecules (Ziegler, Thompson and Cassanovas, 1993), lipid-complexed amylose (Olkku and Rha, 1978), the presence of friction between the swollen granules (Singh et al., 2006), competition between leached amylose and ungelatinized granules for free water (Olkku and Rha, 1978), rotational speed of spindle (Deffernbaugh and Walker, 1989) and harvesting period of the starch source (Liu et al., 2003).

Pasting properties of pulse starches are presented in **Table 2-8**. Differences in techniques used to measure pasting properties among starches, combined with different

Table 2-8: Pasting properties of pulse starches

Starch source	Method (units)	Pasting property					
		Moisture (%w/v)	Pasting Temperature (°C)	Peak viscosity	Breakdown	Final viscosity	Setback
Chickpea[l,c,e,m,n]	RVA (cP)	9.0	69.1-71.8	755-1347	-[2]	1068-1938	320-610
	RVA (cP)	6.0	75.1 - 77.1	1107–2173	-	1639-3250	532-1123
	RVA(mPa/s)	6.0	51.4	3942	897	5541	2496
	BVA (BU)	9.0	75	410-460	-	800-880	240-260
Black Bean[e]	BVA (BU)	9.0-9.45	70 – 75	780-810	-	1200-1360	375-440
Black Gram[m,n]	RVA(mPa/s)	6.0	50.3	5147	1609	4968	1430
	RVA (RVU)	6.0	76.3-80.3	422-514	156-212	400-439	102-134
Cowpea[h]	RVA (cP)	6.0	80.7	1140	-	-	2535
Field Pea[l,m]	RVA(mPa/s)	6.0	52.5	4398	1159	5991	2752
	BVA (BU)	9.0	79.0-79.5	55-70	-	-	230-350
Grass Pea[i,j]	RVA(cP)	7.0	74.1-74.3	3074-3226	491-86	5004-5622	2269-3349
	BVA (BU)	5.0	71.2-75.8	96-103	95-100	140.5-148	-
Kidney Bean[d]	RVA (cP)	11.9	75.2	1980-2286	145-189	4802-6532	3011-4391
Lentil[c,e,m]	RVA (cP)	9.0	70-71.1	1185-1359	140-239	1651-1781	605-662
	RVA(mPa/s)	9.0	50.3	4637	1602	5965	2930
	BVA (BU)	9.0	72	540-560	-	900-920	270-305
Jack Bean[k]	BVA (BU)	8.0	84	645	95 BU	-	875 BU

Starch Source	Method (units)		Pasting property				
			Pasting Temperature (°C)	Peak viscosity	Breakdown	Final viscosity	Setback
Lima Bean[b]	BVA (BU)	9.0	87	668	56	800	188
Mung Bean[f,m]	BVA (BU)	6.0	80	200	-	360	140
	RVA(mPa/s)	6.0	50.2	6107	2523	4779	1195
Navy Bean[d,e]	BVA (BU)	9.0	70 – 72	400-410	-	800-810	190
	RVA (cP)	11.9	73.9	2746	891	5343	3488
Pigeon Pea[g,m]	BVA(BU)	6.0	89	80	-	360	
	RVA(mPa/s)	6.0	50.9	4025	967	5940	2882
Pinto Bean[e]	BVA (BU)	9.5	80 – 82	170-190	-	500-510	500-510
Smooth Pea[e]	BVA (BU)	9.0	74 – 75	300-330	-	720-770	300-330
Velvet Bean[a]	BVA (BU)	9.0	79.5	256	20	350	94
Yellow Pea[h]	RVA (cP)	6.0	70.5	724	-	-	643

[1]Letter superscript indicates the reference: [a]Betancur-Ancona et al., 2002, [b]Betancur-Ancona et al., 2001,[c]Chung et al., 2008a, [d]Chung et al., 2008c [e]Hoover & Ratnayake, 2002, [f]Hoover et al., 1997, [g]Hoover et al., 1993, [h]Huang et al., 2007a, [i]Jayakody et al., 2004, [j]Korus et al., 2008, [k]Lawal and Adebowale, 2005, [l]Ratnayake et al., 2001, [m]Sandhu & Lim, 2008, [n]Singh et al., 2004b

[2] – Indicates not reported

units, make comparisons among different types of pulse starches, as well as cultivars within pulses, difficult. In general, most pulse starches exhibit a high pasting temperature, the absence of peak viscosity, increased viscosity during the holding period, and a high setback (Hoover et al., 2010). These pasting properties are likely reflective of the high amylose content, the presence of only trace amounts of lipid complexed amylose chains, strong interactions between starch chains (amylose-amylose and/or amylose-amylopectin), the orientation of the amylose chains relative to one another, and by the chain lengths of amylose and amylopectin (Hoover et al., 2010; Hoover and Sosulski, 1985).

2.5.5 Retrogradation

Retrogradation occurs when the molecules of gelatinized starch begin to re-associate forming an ordered structure or gel. This process is outlined in **Figure 2-14** whereby, native starch granules (**Figure 2-14i**) heated in excess water above their gelatinization temperature undergo irreversible swelling (**Figure 2-14iia),** which results in amylose leaching into the solution and partial granule disruption forming a starch paste (**Figure 2-14iib**). Upon cooling, the amylose and amylopectin in the gelatinized paste interact, forming a more structured gel structure (**Figure 2-14iii**). The ordered structure can be short-term via amylose crystallization or long-term which is a much slower process involving recrystallization of the amylopectin outer branches (Karim, Norziah and Seow,

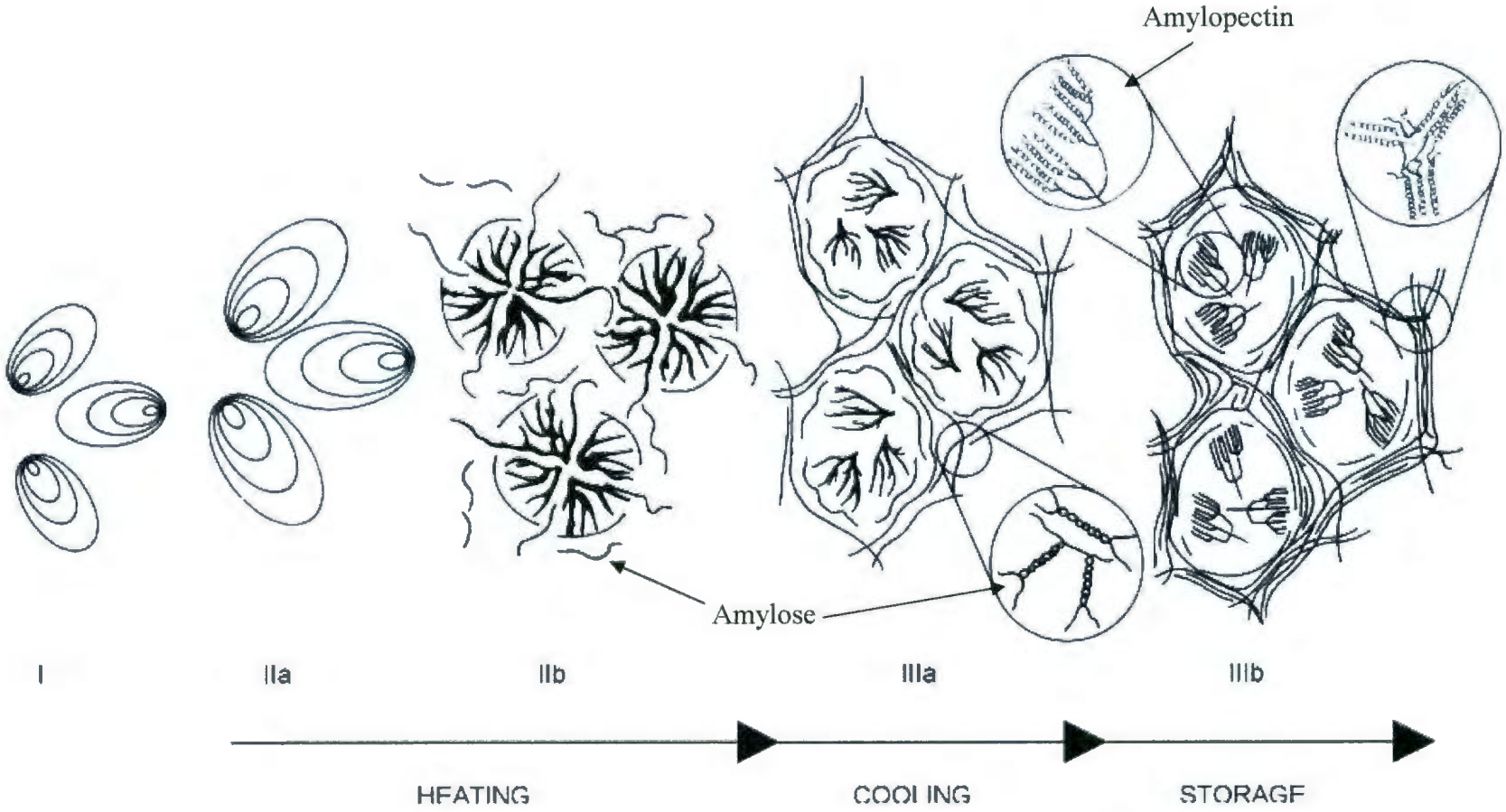
Figure 2-14: Representation of changes that occuring in a starch-water mixture during heating, cooling and storage demonstrating:

i) Native Starch granules

ii) Gelatinization associated with swelling

iii) Cooling of the starch granule during storage

Adapted from Goesaert et al. (2005) with permission from Elsevier


Amylopectin
Amylose
I
IIa
IIb
IIIa
IIIb
HEATING
COOLING
STORAGE

2000). This retrogradation process is both a time and temperature dependant interaction (Hoover and Sosulski, 1991), which is accompanied by an increase in the degree of crystallinity and gel firmness, water exudation (syneresis) and the appearance of a B type X-ray pattern (Hoover, 1995 and Manual; Hoover et al., 2010; Miles et al., 1985).

Starch retrogradation is of great interest in the food industry since it profoundly affects the quality, acceptability, and shelf-life of starch-containing foods (Karim, Norziah and Seow, 2000). In fact, it can be desirable, although retrogradation is mostly considered an unfavorable occurrence since it produces an unfavorable texture in starch foods. Starch retrogradation is the primary process responsible for the staling of bread and other baked products (Eliasson and Gudmundsson, 1996), as well as for syneresis of frozen food (Swinkles, 1985), increased tendency to form stiff gels (Swinkles, 1985), and decreased starch digestibility. However, starch retrogradation is sometimes promoted to modify the structural, mechanical or organoleptic properties of certain starch-based products (Karim, Norziah and Seow, 2000).

2.5.5.1 Mechanism of retrogradation

Retrogradation occurs when the starch gels become rigid and crystallize quickly due to amylose gelation and increased mobility of smaller amylopectin fragments (Biliaderis, 1998; Zhang and Jackson, 1992). This is followed by a further slow crystallite development in the amylopectin region of the starch (Biliaderis, 1998). According to the polymer crystal growth theory, retrogradation occurs via a three step

mechanism: nucleation, propagation or crystal growth, and maturation or crystal perfection (Slade and Levine, 1987).

The nucleation stage involves the formation of crystal nuclei which occurs at the junction points between two or more glucan chains (Slade and Levine, 1987). This initial stage is temperature sensitive since some degree of super cooling below the crystallization temperature is required for crystallite formation. Therefore, the retrogradation process starts with starch storage at approximately 5°C for 24 hours, during which time gelatinized starch molecules begin to re-associate in a less stable configuration than the native starch (Gidley, 1987)

During the propagation stage, there is a continued growth of crystals from the nuclei via intermolecular interactions (primarily hydrogen bonds). This occurs when the starch is stored over a period of time at room temperature. In addition, studies have shown that higher propagation temperatures of up to 40°C correlate positively with increased onset temperatures of retrogradation, but negatively with the crystallite melting temperature range (Jankowski and Rha, 1986; Silverio et al., 2000). As well, increased temperatures rather than increased storage periods produce more symmetrically perfect and stable crystallite structures (Longton and LeGrys, 1981).

The final stage of starch retrogradation is the maturation stage which involves even more continued growth of crystals and perfection via annealing of the microcrystallites (Slade and Levine, 1987). Increased temperatures are positively correlated with a higher maturation rate, but only up to the maximum crystalline temperature (Slade and Levine, 1987).

Both the amylopectin and amylose components of the starch are involved in retrogradation although it has been shown that linear amylose molecules tend to more readily re-associate and form hydrogen bonds than the larger amylopectin molecules (Thomas and Atwell, 1999). Moreover, amylose chains are thought to form double helices of 40-70 glucose units during retrogradation, whereas retrogradation of amylopectin occurs by the association of its outermost branches (Hoover, 2001). The molecular interactions during retrogradation are mainly hydrogen bonding between starch chains, which develop a "B" type crystallinity pattern regardless of the initial crystallinity pattern of the starch (Zobel, 1988; Russell, 1987). These retrograded starches also display both amorphous and crystalline regions (Hoover, 2001).

2.5.5.2 Factors affecting retrogradation

Numerous factors affect the extent and rate of retrogradation. These include, but are not limited to botanical source (Jacobson and BeMiller, 1998), storage temperature (Jankiwski and Rha, 1986), moisture content of the starch (Zelzenak and Hoseney, 1986), starch concentration (Longton and LeGrys, 1981; Liu and Thompson, 1998), initial heating temperature (Liu and Thompson, 1998), lipid content (Keetels et al., 1996; Huang and White, 1993), presence of solutes (salts and sugars) (Ward, Hosney and Seib, 1994; Bello-Perez and Paredes-Lopez, 1995), starch modifications, both physical and chemical (Gunaratne and Hoover; 2002; Perera and Hoover; 1998), and starch structure of amylose and amylopectin (Jacobson and BeMiller, 1998; Russell, 1987). In the case of starch

structure, retrogradation varies depending on the chain length distribution of amylopectin (Thygesen, Rumsden and Cork, 2003; Gudmundsson, 1994), amylose content (Thygesen, Rumsden and Cork, 2003; Singh et al., 2006; Singh, Sandhu and Kaur, 2004), molecular size of amylose (Liu et al., 1997) and amylose to amylopectin ratio (Jacobson and BeMiller, 1998).

2.5.5.3 Methods for measuring retrogradation

There are several methods for measuring the retrogradation of gelatinized starch. However, since retrogradation is a complex process affected by many factors, it is unlikely that any single method would be able to give a complete picture of the retrograded starch gels at both the macroscopic and microscopic levels (Karim, Norziah and Seow, 2000). These methods vary in the retrogradation variable they measure as well as in the information they provide. Some methods to measure retrogradation include, but are not limited to turbidity, which correlates increased retrogradation with decreased light transmittance through the gelatinized starch and measures the precipitation of insoluble starch aggregates (Jacobson, Obanna and BeMiller, 1997; Swinkles, 1985), DSC, monitoring the extent and rate of retrogradation by measuring enthalpy changes in the reformed crystallites (Jayakody et al., 2005), X-ray diffraction, which monitors the crystalline structure for the retrograded gels and also measures the changes in the polymorphic crystalline pattern during retrogradation (l'Anson et al., 1988), Fourier transform infrared spectroscopy (FTIR) using band narrowing to measure

the extent of molecular order (Wilson et al., 1991), proton nuclear magnetic spectroscopy (^{1}H NMR) determining changes in molecular mobility (Wu and Eads, 1993), freeze thaw stability measuring the occurrence of syneresis (Yuan and Thompson, 1998), starch hydrolysis by acid or enzymes which measure starch resistance to forms of hydrolysis (Sievert, Czuchajowska and Pomeranz, 1991) and rheological methods which monitor gel firmness and thus measure changes in visco-elastic properties (Gudmundsson, 1994; l'Anson et al., 1988) .

Retrogradation of pulse starches has been studied mainly using the amount of water exuded when a frozen gelatinized starch gel is thawed at room temperature (syneresis), with DSC, turbidity, X-ray, and NMR studied to a lesser extent (Hoover et al., 2010). Because of the wide array of different techniques used to measure the extent of retrogradation, comparisons of retrogradation or retrogradation patterns among pulse starches is nearly impossible. For example, syneresis data and turbidity measurements provide information about both the amylose and amylopectin crystallization, whereas DSC and NMR provide information on amylopectin crystallization and changes in water mobility during retrogradation, respectively (Hoover et al., 2010). However, based on syneresis data, studies have shown that pulse starches retrograde to a greater extent than cereal or tuber starches, which is likely indicative of their higher amylose content and/or molecular structure (Hoover et al., 2010).

2.5.5.3.1 Turbidity

Increased turbidity is characteristic of aging gelatinized starch solutions, which results from changes in density distribution due to phase separation (Karim, Norziah and Seow, 2000). In starches, turbidity development during storage has been attributed to many factors including granule swelling and remnants, leached amylose and amylopectin which can scatter a significant amount of light, amylose and amylopectin chain length, intra- or intermolecular bonding, lipid and cross-linking substitution (Jacobson, Obanni and BeMiller, 1997; Singh, Sandhu and Kaur, 2004; Perera and Hoover, 1999). Aggregation and crystallization of amylose have been reported to be complete within the first few hours of storage, whereas those of amylopectin occur during later stages (Singh and Singh, 2001).

Turbidity is used to characterize the behavior of starch precipitation when cooked, which as a result, can determine the behavior of the linear amylose content (Adebooye and Singh, 2008). In fact, turbidity development results from molecular associations that occur during the early stages of the retrogradation process, before larger-scale organizations (that are more easily detected using other methods) are formed (Karim, Norziah and Seow, 2000).

Several factors influence the starch turbidity including: amylose content (turbidity increases with amylose content) (Yu et al., 2012), the amount of amylose complexed with other molecules such as lipids and phosphorous, whereby the complexes retard the starch molecule aggregation resulting in decreased turbidity (Yu et al., 2012), and granule size, where larger sized granules have lower turbidity than smaller ones (Singh and Singh,

2001). Starches may also contain varying amounts of phosphate monoester derivatives that increase paste clarity and viscosity (Jane, Kasemsuwan and Chen, 1996).

Currently, there is a dearth of information on the use of turbidity as a comparative method for assessing the extent of pulse starch retrogradation. However, it is known that the turbidity of starches increases progressively over time (Sandhu, Singh and Kaur, 2004).

2.5.6 Starch Hydrolysis

2.5.6.1 Acid Hydrolysis

Acid hydrolysis has been used for many years to modify the starch granule structure to produce "soluble starch". Starches treated with sulfuric acid (15% w/v) produce a mixture of low molecular weight, linear, and branched dextrins with an average degree of polymerization (DP) of 25-30, known as Nägeli amylodextrins, while starches treated with an aqueous solution of hydrochloric acid (7.5% w/v) produce a high molecular weight starch, known as lintnerized amylodextrins (Hoover, 2000).

Acid hydrolyzed starches have several industrial applications, including but not limited to its use as a pre-modification step for the production of cationic and amphoteric starches, as a wrap sizing agent to increase yarn strength and abrasion resistance in the weaving industry, for the preparation of starch gum candies, for the manufacture of

gypsum board for dry wall construction, and for the manufacture of paper and cardboard (Solarek, 1987).

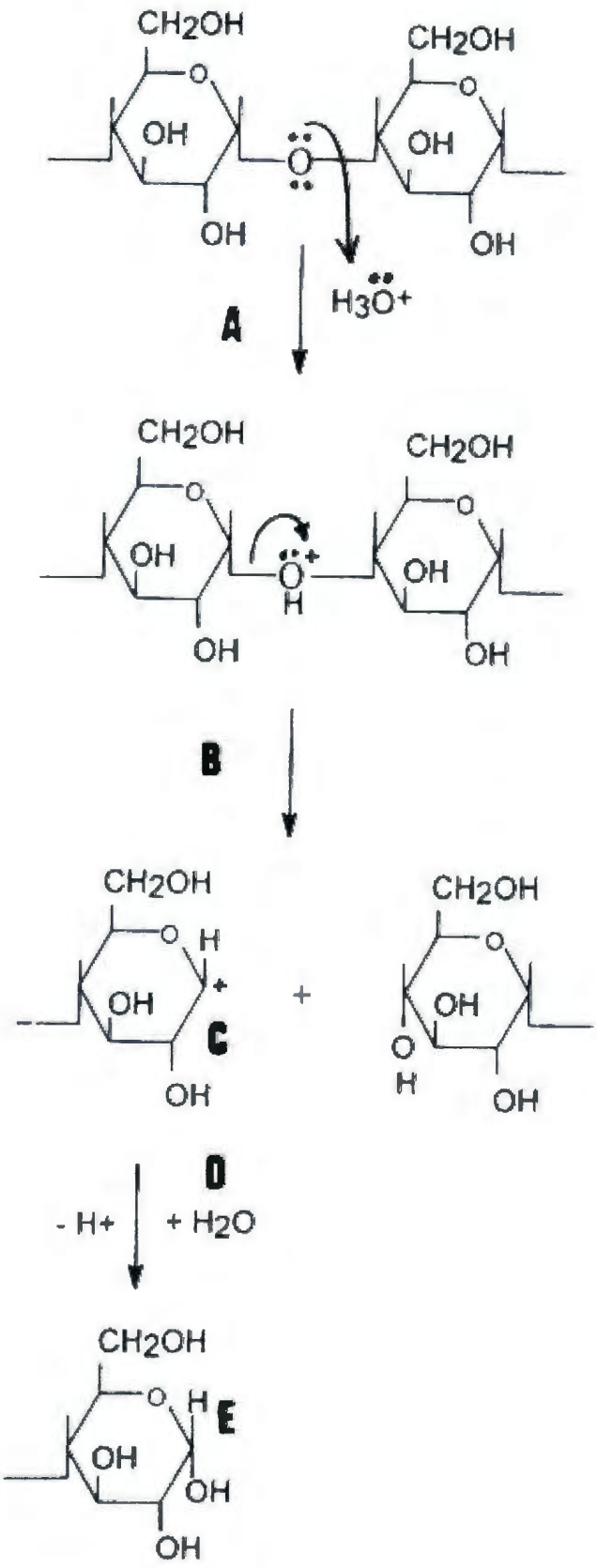
2.5.6.1.1 Mechanism of acid hydrolysis

When starch is subjected to acid, it attacks both the α-(1→4) and α-(1→6) glycan linkages. As described by Hoover (2000), the hydronium ion (H_3O^+) from the acid, carries out an electrophillic attack on the oxygen atom of the glycosidic bond, as demonstrated in **Figure 2-15A**. This is followed by the electron in one of the carbon-oxygen bonds moving onto the oxygen atom (**Figure 2-15B**), generating an unstable, high-energy carbocation intermediate (**Figure 2-15C**). This carbocation intermediate is a Lewis acid which in the presence of water, results in the regeneration of a hydroxyl group (**Figure 2-15E**)

All starches are known to display a two-stage hydrolysis pattern in the presence of acid, whereby there is a relatively fast hydrolysis reported during the first 8 days, followed by a slower hydrolysis rate beyond day 8 (Hoover, 2000; Hoover and Vasanthan, 1994; Vasanthan and Bhatty, 1996). The initial period corresponds primarily to the hydrolysis of the amorphous regions of the starch granule, while the second hydrolysis stage corresponds to the hydrolysis of the crystalline regions (Hoover, 2000; Jayakody et al., 2007). The slower hydrolysis of the crystalline parts of the starch granule regions has been explained by two hypotheses (French, 1984; Kainuma and French, 1971). They postulate that: 1) the dense packing of starch within the starch crystallites does not readily allow the penetration of the acid (H_3O^+) into the starch

Figure 2-15: Mechanism of acid hydrolysis of starch

Adapted from Hoover (2000) with permission from Taylor & Francis


CH2OH
OH
OH
CH2OH
OH
OH
H3O+
A
CH2OH
OH
OH
CH2OH
OH
OH
H
B
CH2OH
H
OH
C
OH
CH2OH
OH
O
H
OH
D
- H+
+ H2O
CH2OH
H
E
OH
OH
OH

regions, and 2) acid hydrolysis of a glycosidic bond may require a conformational change (chair to half-chair) of the D-glucopyranosyl unit which would be sterically hindered in the crystalline region (Hoover, 2000).

Wang et al. (2012) suggested that in pea starch, acid hydrolysis occurs within 1 day on the surface of the granule and involves both amylose and amylopectin, whereby the degradation of amylopectin occurs mainly at the α-(1→6) branch points in the amorphous lamellae. This was followed by acid attack on predominantly the amylose fraction, resulting in an increase in the degree of crystallinity due to the faster degradation of the amorphous region. In the later stages of hydrolysis, the crystalline regions are degraded simultaneously with the amorphous regions, resulting in only small changes in the crystallinity (Wang et al., 2012).

2.5.6.1.2 Factors affecting acid hydrolysis

In general, B-type starches, such as tuber and roots starches are more resistant to acid hydrolysis than the A-type cereal starches (Jayakody and Hoover, 2002, Jane Wong and McPherson, 1997). However, there is a dearth of information on the acid hydrolysis pattern of pulse starches since pulses display the C type crystallization pattern. Therefore, it is likely, that the susceptibility of the pulse starch to acid is dependent on the fraction of A and B crystallinity patterns exhibited by the starch. Thus, the extent of acid hydrolysis of pulse starches could also be dependent on the B polymorphic content of the starch, as well as the amylopectin branched chain length distribution.

Jane (2006) proposed two mechanisms to explain the protection of branch chain linkages from acid hydrolysis, which is likely due to the position of the glycan linkages of the amylopectin molecules: Mechanism (1) - In A-type starches, the amylopectin branch linkages are more scattered due to large proportions of A and B_1 chains relative to the long B-chains since the α-(1→6) linkages have to be more scattered to accommodate the number of branched linkages. When the branch linkages are scattered in the amorphous and crystalline regions (mainly in A-type starches), those in the amorphous regions are easily hydrolyzed by H_3O^+ and therefore produce linear chains, whereas those located in the crystalline regions are protected and remain as branched chains (Jane et al., 1997; Jane, 2006). Conversely, for the B-type starches, there are fewer short chains relative to the long B-chains. Thus the branch linkages are present in a cluster and located in the amorphous region. Consequently, the branch linkages are easily hydrolyzed by H_3O^+ and produce linear chains. Mechanism (2) – In A-type starches, the A-type polymorphic unit cell (monoclinic) is tightly packed, whereas, in B-type starches, the hexagonal unit cell is relatively loosely pacjed with an open channel of water in the unit cell. Consequently, the closely packed A-type uinit cell would hinder acid hydrolysis of its α-(1→6) branch linkages and procuce branched (Jane, 2006).

The hydrolysis of starches in the presence of acid is influenced by a number of other factors. Some of these factors include, but are not limited to: starch source (Srichuwong et al., 2005; Hoover and Vasanthan, 1994; Hoover, 2001), granule size (Jayakody and Hoover, 2002), presence of pores on the starch granule surface (Jayakody and Hoover, 2002), granular swelling (Jayakody et al., 2005), starch damage (Tester,

Debon, and Karkalas, 1998), amorphous layer characteristics (Srichuwong et al., 2005) including the degree of association of the starch chains within the amorphous region (Hoover, Swamidas and Vasanthan, 1993), as well as properties of the starch amylose and amylopectin components. The latter includes factors such as amylose content (Jayakody and Hoover, 2002), lipid-complexed amylose (Jayakody and Hoover, 2002; Hoover, 2000) amylopectin structure (Srichuwong et al., 2005), relative crystallinity (Jayakody et al., 2005) as well as the proportion of B-type crystallites (Srichuwong et al., 2005).

2.5.6.2 Enzyme Hydrolysis

Enzyme and acid hydrolysis have been widely used to modify native starches to create products with altered solubility, viscosity, and/or gelation properties that have numerous applications in the food, paper, textile, and other industries (You and Izydorezyk, 2007). However, in contrast to acid hydrolysis, α-amylases, the key enzyme responsible for starch hydrolysis, can cleave only the α-(1→4) glucosidic bonds and not the α-(1→6) glucosidic bonds, thus producing smaller chains of oligosaccharides having the α-configuration at the C1 of the reducing glucose unit (Ao et al., 2007). In addition, compared to the hydrochloric acid hydrolysis, α-amylase is relatively large in size (6 nm in diameter), and thus cannot easily diffuse into the granule.

Aside from the α-amylase, the amyloglucosidase enzyme also assists in starch hydrolysis, whereby the α-amylase controls the rate of starch hydrolysis, while the

amyloglucosidase converts amylase degradation products to glucose and prevents the inhibition of α-amylase activity (Zhang, Ao, and Hamaker, 2006).

2.5.6.2.1 Mechanism of α-amylase attack

α-Amylase catalyses the hydrolysis of the α (1→4) glycosidic bonds in amylose and amylopectin, whereby it is proposed to have a multiple attack mechanism, starting at the reducing end to the non-reducing end (Hoover and Zhou, 2003). In this mechanism, once the enzyme forms a complex with the substrate and forms the first cleavage, the enzyme remains with one of the fragments of the original substrate and catalyses the hydrolysis of several bonds before it disassociates and forms a new active complex (Robyt and French, 1970).

Porcine pancreatic α-amylase has five binding sites with the catalytic site located between subsites 2 and 3, with two subsites to the right and three subsites to the left. Only the chain to the right diffuses away after initial cleavage and the remaining chain to the left diffuses to fill the open binding subsites to maltose, maltotriose, and maltotetraose as products in a multiple attack mechanism, which in particular maltose and maltotriose, are known to have an inhibitory effect on the action of α-amylase in vitro. Maltose and maltotriose bind strongly to pancreatic α-amylase, which inhibits their absorption onto the crystalline spherulites of short chain amylose (Zhou, Hoover and Liu 2004). In order for cleavage of an α-(1→4) glycosidic linkage, α-amylase must bind to at least three glucose units (Zhang, Ao and Hamaker, 2006).

There still remains some controversy over how α-amylase attacks the amorphous or the crystalline regions of the starch granule. Presently, there are two proposed mechanisms of α-amylase attack on the starch molecule: 1) inside-out digestion (**Figure 2-16B**), and 2) side-by-side digestion (**Figure 2-16C**).

Some studies suggest that hydrolysis by α-amylase initially occurs at the granule surface whereby α-amylase makes a depression on the peripheral region of the granule, creating a pore, and then penetrates deep into the granule interior during subsequent attack (Hoover et al., 1997). The internal sides of the pores and channels then become active sites for the enzyme. Since the alignment of double helices formed by amylopectin in the crystalline regions is perpendicular to the starch granule surface, α-amylase can bind the starch molecules in a parallel, or side-by-side, direction to the double helices (Zhang Ao and Hamaker, 2006). Thus, enzyme hydrolysis from the sides of the crystalline lamellae enlarges the internal channels, resulting in granule fragmentation (Oates, 1997). This is known as side-by-side digestion mechanism in which both the amorphous and crystalline regions are digested simultaneously at the same rate (Lauro et al., 1999; Zhang, Ao and Hamaker, 2006).

Others suggest that α-amylase hydrolysis is initiated in the helium region of the starch granule and continues towards the outside, known as the inside-out digestion mechanism. Inside-out digestion occurs at high amylopectin concentration and tightly packed chains in the granule which presents a resistant surface thus resulting in a slow rate of hydrolysis, whereas the inside-out pattern proceeds rapidly from the granule interior (Oates, 1997). Some, however, believe that the inside-out digestion is a different

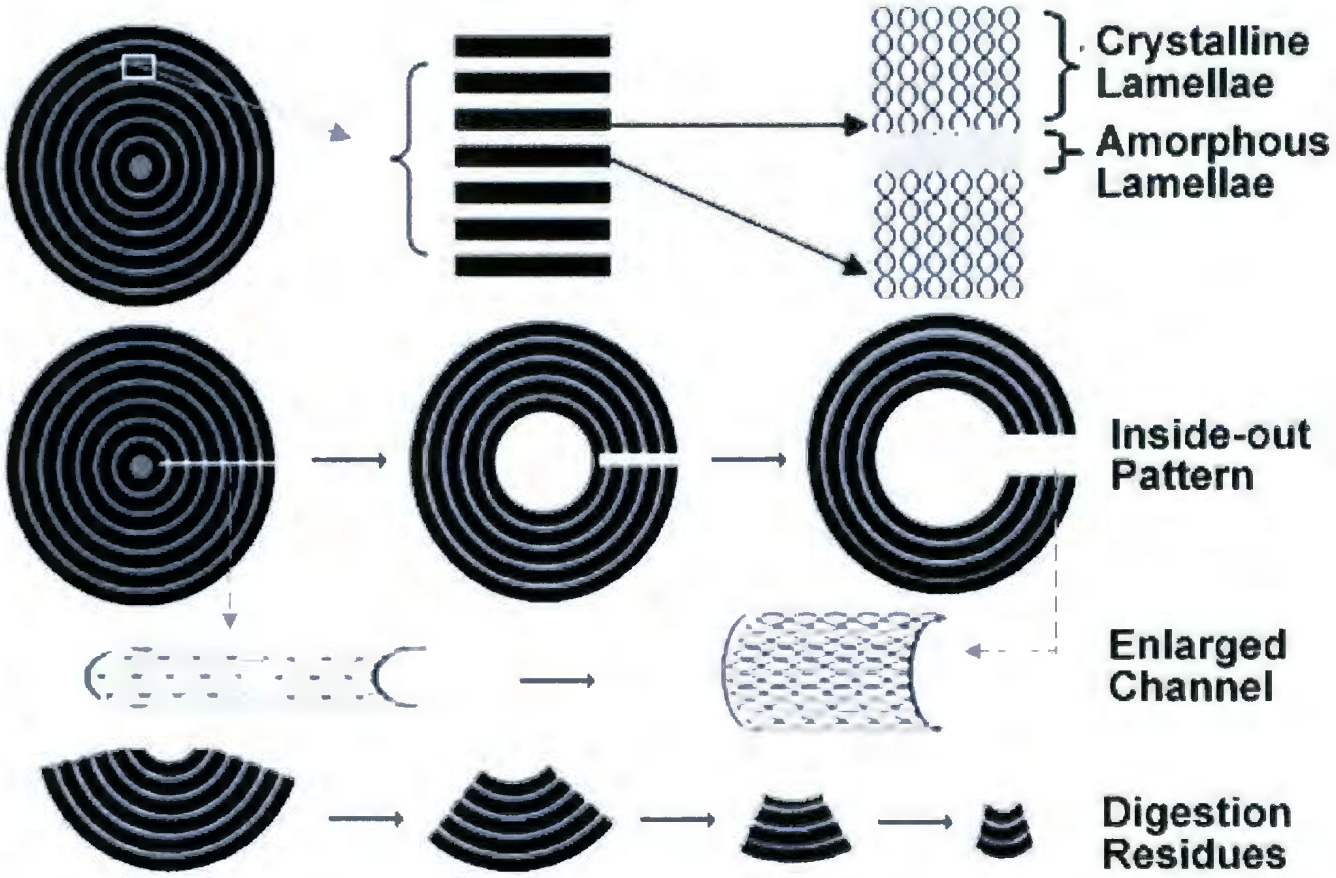
Figure 2-16: Mechanism of α-amylase enzymatic hydrolysis demonstrating:

a) Crystalline and amorphous layer structure

b) the inside-out layer-by-layer digestion

c) side-by-side digestion

Adapted from Zhang, Ao and Hamaker (2006) with permission from the American Chemical Society


Crystalline Lamellae
Amorphous Lamellae
Inside-out Pattern
Enlarged Channel
Digestion Residues

projection of the side-by-side digestion (Zhang, Ao and Hamaker, 2006).

2.5.6.2.2 Factors affecting α-amylase hydrolysis

There are several factors that can influence the rate and extent of α-amylase attack on starch granules. These include: botanical source (Gudmundsson and Eliasson, 1993) the packing of B-type crystallites within the granule (Gerard et al, 2001), amylose/amylopectin ratio (Hoover and Sosulski, 1985), extent of packing of amylose and amylopectin at the granule surface (Jane 2006; Zhang, Ao and Hamaker, 2006), degree of crystallinity (Hoover and Sosulski, 1985), extent of crystallite perfection (Zhang, Ao and Hamaker, 2006), amylose-lipid complexes (Hoover and Manual, 1995), granule size (Snow and O'Dea, 1981; Cottrell et al., 1995), granule size distribution (Zhang, Ao and Hamaker, 2006; Vasanthan and Bhatty, 1996) surface porosity (Huber and BeMiller, 1997; Zhang, Ao and Hamaker, 2006), and extent of association of molecules between starch components (Dreher, Berry and Dreher, 1984; Zhou, Hoover and Liu, 2004).

The extent of α-amylase hydrolysis is also affected by polymorphic form in which A- and B-type starches have different susceptibilities, with A-type being more susceptible (Jane, Wong and McPherson, 1997, Zhang, Ao and Hamaker, 2006). In the case of A-type starches, the branch points are scattered in both amorphous and crystalline regions, thus there are many short A-chains of amylopectin that are derived from branch linkages located inside the crystalline region. This produces an inferior crystalline structure which contains α-(1→6) linked branch points and short double helices which are more

susceptible to α-amylase hydrolysis. These are known as weak points in the A-type starches which are readily attacked by the α-amylase enzyme (Jane, Wong and McPherson, 1997). However, since the B-type starches have more branch points located in the amorphous region, combined with fewer short branch chains of amylopectin, the crystalline region of B-type starches are more resistant to α-amylase attack (Jane, Wong and McPherson, 1997).

Native pulse starches are known to be more digestible than potato or high amylose maize starch (B-type), but less digestible than cereal starches (A-type) (Hoover et al., 2010). The reduced digestibility of pulse starches has been attributed to the absence of pores on the granule surface (Hoover and Sosulski, 1985), high amylose contents (Hoover and Zhou, 2003), strong interactions between amylose chains (Hoover and Sosulski, 1985), and the number of B-crystallites (Hoover and Zhou, 2003), whereby in the latter case, resistance to α-amylase attack increases proportionally with increase in the 'B' polymorph content (Ratnayake et al., 2001).

2.5.7 Starch digestibility

Within the body, the digestibility of starch is measured using two components: kinetics and the completeness of the starch's digestibility (Sandhu and Lim, 2008). Differences in the digestibility of starches among various starch species can be attributed to a combination of many factors including starch source (Ring et al., 1988), amylose/amylopectin ratio (Hoover and Sosulski, 1985) , amylose content (Hoover and

Zhou, 2003), degree of crystallinity (Chung, Lim and Lim 2006; Hoover and Sosulski, 1985; Sandhu and Lim, 2008), polymorphic pattern distribution (Jane, Wong, and McPherson, 1997), molecular weight of amylose and amylopectin (Sandhu and Lim 2008; Madhusudan and Tharanathan, 1996), molecular structure of amylopectin (Srichuwong and Jane, 2007), amylose chain length (Jood, Chauhan and Kapoor, 1988), granule size (Sandhu and Lim, 2008; Snow and O'Dea, 1981), distribution of starch in relation to dietary fiber components (Dreher, Berry and Dreher, 1984; Snow and O'Dea, 1981), and the presence of various anti-nutrients, including phenols and phytic acid (Deshpande and Cheryan, 1984; Thomson and Yoon, 1984).

Classification of hydrolyzed starch is based on the rate of glucose release and its absorption into the gastrointestinal tract (Englyst, Kingman and Cummings, 1992). These classifications are known as rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS). Studies have shown that the glycemic indices of food products are positively correlated with the amount of rapidly digestible starch (Zhang, Ao and Hamaker, 2006). In this case, resistant starch and slowly digestible starch result in low glycemic index in starch-based foods (Liu et al., 2006).

Rapidly digestible starch (RDS) is the starch fraction that is digested within 20 minutes of consumption, causing a sudden increase in blood glucose level after ingestion (Chung, Liu and Hoover, 2009; Zhang Ao and Hamaker, 2006). Moreover, slowly digestible starches are hydrolyzed between 20 and 120 minutes, with the remaining starch after 120 minutes classified as resistant starch (Zhang, Ao and Hamaker, 2006).

Slowly digestible starch (SDS) is generally the most desirable form of starch as it is digested completly, but more slowly in the small intestine (Sandhu and Lim, 2008) and attenuates postprandial plasma glucose and insulin levels (Jenkins et al., 1981). SDS maintains blood glucose levels over a period of time in comparison to RDS which causes a fast and high peak in glucose levels which declines quickly below baseline (Lehmann and Robin, 2007). Thus, SDS does not produce a hyperglycemic state, followed by a hypoglycemic one (Güzel and Sayar, 2010). Health benefits of SDS include stable glucose metabolism and diabetes management, increased mental performance, and increased satiety (Lehmann and Robin, 2007).

Resistant starch (RS) has been defined as the fraction of starch that escapes the small intestine, and has functional and nutritional properties in conjunction with dietary fiber, such as not releasing glucose into the bloodstream (Brown, 2004; Themeier et al., 2005). Resistant starch is fermented by the natural microflora of the colon to produce short chain fatty acids (Mahadevamma and Tharanathan, 2004). Although resistant starch is present in native legume starch, it can also be formed during the processing of starch. This formation of resistant starch is dependent on numerous factors, primarily the starch composition, including amylose/amylopectin ratio, starch-protein interactions, amylose-lipid complexes and the rate of starch retrogradation (Mahadevamma and Tharanathan, 2004). There are numerous reported health benefits for RS including the prevention of colon cancer, hypoglycemic effects, substrate growth of probiotic microorganisms, reduction of gall stone formation, hypocholesterolemic effects, inhibition of fat accumulation, increased mineral absorption, and prevention of

constipation, cholera, and osteoporosis (Brown, 2004; Sajilata, Singhal and Kaularni, 2006). Many of these effects are believed to be related to the fermentation abilities and probiotic properties, in which the latter stimulates the growth of beneficial bacterial in the colon (Brown, 2004).

Table 2-9 summarizes the relative amounts of digestible starch in pulses. Unfortunately, comparisons among different pulses is restricted because of both the limited number of starches studied and differing methodologies used, which differ according to the enzymes used and time of hydrolysis (Hoover, 2010). However, according to **Table 2-9**, all pulse starches have rapidly digestible starches present in the smallest proportion, which could help explain why their consumption does not cause a sudden increase in blood glucose levels. In general, cereal starches have more rapidly digestible starch than legume and tuber starches, with tuber starches showing the highest amount of resistant starch (Liu et al., 2006).

Table 2-9: Rapidly digestible, slowly digestible, and resistant starch content of pulse starches

Starch source	Method	Digestible Starch (%)		RS (%)
		RDS[2]	SDS	
Chickpea[1,a,c,d]	Englyst	10.9	34.8	54.3
	AACC	9.4-12.4	27.1-30.7	3.1-6.4
	Englyst	19.7-23.5	45.7-41.5	33.5-35.0
Black Gram[d]	Englyst	9.5	29.6	60.9
Field Pea[d]	Englyst	8.1	33.9	58.0
Lentil[a,d]	Englyst	5.2	29.7	65.2
	AACC	7.6-7.8	23.7-24.7	14.4-14.9
Kidney Bean[b]	AACC	11.7	65.7	17.2
Mung Bean[d]	Englyst	9.7	40.0	50.3
Pigeon Pea[d]	Englyst	4.2	16.9	78.9
Smooth Pea[a]	AACC	9.2-10.7	23.3-26.5	10.1-14.7

[1]Letter superscript indicates the reference: [a]Chung et al., 2008a, [b]Chung et al., 2008c, [c]Miao, Zhang & Jiang, 2009, [d]Sandhu & Lim, 2008.

[2]RDS, SDS, and RS represents rapidly digestible starch, slowly digestible starch, and resistant starch, respectively.

Chapter 3: Materials and Methods

3.1 Materials

Chickpea (*Cicer arietinum L.*) cultivars (CDC Xena, CDC Flip 97-133c, CDC 418-59, CDC ICC 12512-9) were obtained from the Crop Development Center at the University of Saskatchewan, Saskatoon, SK, Canada, and grown in Kyle, Saskatchewan under identical conditions. Fungal α-amylase (39.3 units/mg solid) and crystalline pancreatic porcine α-amylase (1122 units/mg solid) from *Aspergillus oryzae* were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All chemicals and solvents were of ACS certified grade.

3.2 Methods

3.2.1 Starch Isolation

Starch was isolated from the chickpea seeds following the procedure of Hoover and Sosulski (1985). The chickpea seeds were steeped in 0.01% sodium metabisulfite (300mL) for 24 hours at a temperature range of 30-35°C to prevent microbial spoilage. The swollen seeds were then washed thoroughly, peeled, and homogenized in a Commercial Warring Blender (Dynamics Corporation of America, New Hartford, CT, USA) for 3 intervals of one minute each. The homogenate was filtered through a double

layer of cheesecloth (under vacuum), and the filtrate collected and allowed to settle overnight. The supernatant was then removed using a siphoning tube, and the sediment suspended in excess 0.2% (w/v) NaOH, which was removed after 12 hours. The sedimentation procedure was repeated three times. Next, the sediment was suspended in water and filtered under vacuum through a 70μm polypropylene filter. The filtrate was allowed to settle for 2 hours, and the supernatant was then removed by centrifugation (1000 x g). This procedure was repeated thrice. Finally, the starch slurry was neutralized with HCl (0.02N) to a pH of 7.0. The neutralized solution was filtered through a double-layer of Whatman No. 4 filter paper, and air dried gradually over a 48 hour period. The dried cake was crushed manually and passed through a 250μm test sieve (Fisher Scientific Company, Pittsburgh, PA, USA) to obtain a free-flowing powder. The powder was finally weighed and the yield calculated as the percentage of the initial chickpea seed weight.

3.2.2 Granule Size Estimation and Morphology

3.2.2.1 Granule morphology

Granule morphology of native chickpea starches was studied using scanning electron microscopy (SEM). Starch samples were sieved through a 63 μm mesh sieve and then were mounted on Cambridge-type circular aluminum stubs containing carbon electro-conductive adhesive tape (Electron Microscopy Science, Hatfield, PA, USA).

The starches were then coated with 20nm of gold and examined and photographed using a Hitachi scanning electron microscope (S570, Nissei Sangyo Inc., Rexdale, ON, Canada) at an accelerating potential of 12kV.

3.2.2.2 Starch granule size determination

The size of starch granules from the four chickpea cultivars were analyzed by the following method. Purified starch granules were suspended in water (1mL). A drop of the starch suspension was then spread on a microscope slide, covered with a coverslip and sealed with nailpaint. The slide was then placed on the stage of a Zeiss (Axiophot) microscope, and an image analyzer equipped with image acquisition and processing software (Northern Eclipse 6.0, Empix Imagine Inc, Mississauga, ON, Canada) was used to analyze images of approximately 4000 starch granules from each of the chickpea cultivars at a 20x magnification of the lens. Starch granules were grouped according to their diameters and the number of starch granules in each group was counted. The frequency (%) of starch granules were plotted against granule diameters.

3.2.3 Compositional Analyses

3.2.3.1 Moisture content

Moisture contents of the chickpea starch samples were determined according to the AACC (American Association of Cereal Chemists, 1984) method. Pre-weighed

samples of starch were dried in aluminum pans in a forced air oven (Fisher Isotemp 615G, Fisher Scientific, Nepean, ON, Canada) at 130 ± 1°C for 1 hour. After heating, the starch samples were cooled in a dessicator and then the moisture content calculated as the percentage of weight loss due to drying.

3.2.3.2 Nitrogen content

The micro-Kjeldahl method (AACC, 2000) was used to quantify the amount of nitrogen in the chickpea cultivars. Starch samples (0.3g, db) were weighed on nitrogen-free weighing paper and placed into digestion tubes on a Buchi 430 digester (Buchi Laboratorimus-Technik AG, Flawill/Schweiz, Switzerland). Two Kjeltab M pellets and 20mL of concentrated sulfuric acid (two catalysts of the reaction) were added to the tubes, which were digested until a clear yellow solution was obtained. After digestion, the samples were cooled, followed by the addition of 50mL of distilled water and 100mL of 40% (w/v) NaOH. The released ammonia was then steam distilled using a Buchi 321 distillation unit into a 50mL solution of 4% (w/v) of boric acid containing 12 drops of end-point indicator (N-point indicator, EM Science, Gibbstown, NJ, USA). After 150mL of the distillate was collected, the amount of ammonia released was determined by titrating against 0.05N sulfuric acid. The percentage of nitrogen was calculated as:

$$\text{Nitrogen (\%)} = \frac{(\text{Volume of acid} - \text{Blank}) \times \text{Normality of acid} \times 14.0067 \times 100}{\text{Sample weight (mg)}}$$

3.2.3.3 Lipid content

Both the surface and bound lipids were extracted from the chickpea starch samples using the following procedures. The lipids extracted were expressed as percentages of the initial starting mass of the starches.

3.2.3.3.1 Surface lipids

The extraction of surface lipids was carried out at room temperature (23 – 25°C) by adding 100mL of 2:1 (v/v) chloroform-methanol to starch (5g, db) in a flask which was then stirred using a magnetic stir bar for 1 hour. The starch slurry was then filtered (Whatman No. 4 filter paper) into a round bottom flask with the starch reside washed thrice with small aliquots of the chloroform-methanol solution. The filtrate was evaporated to dryness using a rotary evaporator (Rotavapor –R110, Buchi Laboratorimus, Technik AG, Flawill/Schweiz, Switzerland). The crude extracted lipids were purified before quantification using the method of Bligh and Dyer (1959). The starch resides was saved and subsequently used for bound lipid extraction.

3.2.3.3.2 Bound lipids

The residues remaining from surface lipid extraction was used for extraction of bound lipids. The residues was placed inside a cellulose thimble and refluxed in a 3:1 (v/v) n-propanol-water solution at 85°C for 7 hours in a soxhlet apparatus (Vasanthan and Hoover, 1992). The extract was evaporated to dryness using a rotary evaporator and then crude lipid purified using the method of Bligh and Dyer (1959) before quantification.

3.2.3.3.3 Bligh and Dyer (1959) method for lipid purification

Lipid (surface and bound) extracts were purified by extraction with 1:2:0.8 (v/v/v) chloroform-methanol-water and by forming a biphasic system (1:1:0.9, v/v/v chloroform-methanol-water) by the addition of chloroform and water at room temperature (23-25°C) in a separatory funnel. The heavy chloroform layer, which contained the purified lipid, was withdrawn into a pre-weighed 50mL round bottom flask and then evaporated to dryness using a rotary evaporator (at 60°C) followed by drying in a forced-air oven at 60°C for 1 hour. The dried lipid was then cooled to room temperature in a dessicator.

3.2.3.4 Amylose content

Both the apparent and total amount of amylose was determined using the method of Hoover and Ratnayake (2001).

3.2.3.4.1 Apparent amylose content

Chickpea starch (20 mg, db) was accurately weighed into test tubes and then 8mL of 90% dimethylsulfoxide (DMSO) was added to the tube followed by vigorous mixing using a vortex for 2 minutes. The samples were heated for 15 minutes in water bath (PolyScience, Model 2L-M, PolyScience, Niles, IL, USA) at 85°C with intermittent mixing. The tubes were then allowed to cool to room temperature (22±1°C). The starch solution was quantitatively transferred to a 25mL volumetric flask and diluted to the mark with distilled water. A 1mL aliquot of the diluted solution was mixed with water

(40mL) and I_2/KI solution (5mL [0.0025M I_2 and 0.0065M KI]) and vortexed. The final volume was adjusted with distilled water and the contents were allowed to stand in the dark for 15 minutes at room temperature. The absorbance of the samples was measured at 600nm using a UV-visible spectrophotometer (LKB Novaspec-4049 spectrophotometer, LKB Biochrom Ltd., Cambridge, England). Amylose content was calculated from a standard curve of pure potato amylose (0-50%) and amylopectin (100-50%) (see appendix) in order to avoid overestimation of amylose content (due to formation of a complex between I_2 and the outer branch chains of amylopectin).

3.2.3.4.2 Total amylose content

Total amylose contents of starch samples were determined by the above procedure, but with prior defatting with hot n-propanol-water (3:1 v/v) for 7 hours.

3.2.4 Starch Damage

Starch damage was estimated using the method of Jayakody et al. (2005). Phosphate buffer (40mL, 0.02M, pH 6.9) was added to the chickpea starches (1g, db) and fungal α-amylase (2500 Sigma units, 39.3 units/mg solid) in an erlenymer flask. After incubation at 37°C for 15 minutes, 10mL of anhydrous trichloroacetic acid (10%, w/v) was added to stop the reaction. The mixture was allowed to stand for 2 minutes and then centrifuged at 1000 x g for 10 minutes. The supernatants were neutralized to pH 7.0 and the amount of reducing sugars in the supernatants (2.0mL) was determined using the

Somogyi-Nelson method (Nelson (1944), Somogyi (1952)). Controls without starch but subjected to the above experimental conditions were run concurrently. The percentage of starch damage was calculated according to the following equation:

$$\text{Starch damage (\%)} = \frac{M}{Wx1.05} \times 100$$

where: M - mg of maltose equivalents in the digest (50mL)

W- mg of starch (db)

1.05 – molecular weight conversion factor of starch to maltose

3.2.4.1 Somogyi-Nelson method of reducing sugar determination

Materials:

Alkaline reagent: Anhydrous sodium carbonate (25g), sodium potassium tartate (25g), sodium bicarbonate (20g), and anhydrous sodium sulfate (200g) were dissolved in 800mL of distilled water, and the final volume adjusted to 1 liter.

Copper reagent: Cupric sulfate pentahydrate (37.5g) was added to 50mL of distilled water with 1 drop of concentrated sulfuric acid then added.

Arsenomolybdate reagent: Ammonium molybdate (25g) was dissolved in 450mL of distilled water with concentrated sulfuric acid (21mL) added. Sodium arsenate heptahydrate (3g) was separately dissolved in 25mL of distilled water which was then slowly added with constant stirring to the above solution. The solution was then adjusted to a final volume of 500mL and incubated at 37°C for 24 hours.

Method:

A 1mL aliquot of freshly prepared alkaline copper reagent (25 parts alkaline reagent and 1 part copper reagent) was added to 2mL of the starch supernatant in a test tube. The tubes were heated in a boiling water bath for 20 minutes and then cooled rapidly in cold water. 1mL of the arsenomolybdate solution was then added to each tube which was mixed gently and kept for 5 minutes at room temperature for color development. The solution was diluted with 6mL distilled water and the absorbance measured at 510nm. A reagent blank containing 2mL of distilled water instead of the starch solution was also prepared. The amount of maltose equivalents in the sample was estimated using a standard curve constructed from known maltose concentrations (see appendix).

3.2.5 Starch Structure Determination

3.2.5.1 Amylopectin branch chain length distribution

Isoamylase debranching of whole starch accompanied by high pressure anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was used to determine the amylopectin branch chain length distribution of native chickpea starches (Jayakody et al., 2005). Starch was dispersed in 90% dimethylsulfoxide (2mL) at a concentration of 5mg/mL by stirring in a boiling water bath for 20 minutes. After cooling, 6mL of methanol was added and mixed by vortexing. The solution was placed

in an ice bath for 30 minutes, and then centrifuged (1000 x g) for 12 minutes. The supernatant was discarded and the pellet dispersed in 2mL of sodium acetate buffer (50mM, pH 3.5) by stirring in a boiling water bath for 20 minutes. After equilibration of the tube at 37°C, 5μL isoamylase (68 000 μL/mg protein) was added. The samples were then incubated at 37°C for 22 hours with slow stirring. Following incubation, the enzyme was inactivated by boiling for 10 minutes. A 200μL aliquot of the cooled debranched sample was diluted with 2mL NaOH (150mM) and then filtered (0.45μm nylon syringe filter) and injected into the HPAEC-PAD system (50μL sample loop).

The HPAEC-PAD system consisted of a Dionex DX 600 equipped with an ED50 electrochemical detector with a gold working electrode, GP50 gradient pump, LC30 chromatography oven, and an AS40 automated sampler (Dionex Corporation, Sunnyvale, CA, USA). The standard triple potential waveform was employed with period and pulse potential as follows: T_1=0.40s with 0.20s sampling time, E_1=0.05V; T_2=0.20s, E_2=0.75V; T_3=0.40s, E_3=-0.15V. Data were collected using Chromeleon software, version 6.50 (Dionex Corporation, Sunnywale, CA, USA). Eluents were prepared in distilled water with helium sparging; eluent A was 50mM sodium acetate in 150mM NaOH, and eluent B was 150mM NaOH. Linear debranched amylopectins were separated on a Dionex CarboPac™ PA1 analytical column (4 x 50mm) with gradient elution (-5 to 0 minutes, 40% A; 5 minutes, 60% A; 45 minutes, 80% A) at a column temperature of 26°C and a flow rate of 1mL/min. A CarboPac™ PA1 guard column (4 x 250mm) was installed in front of the analytical column.

3.2.5.2 Wide angle X-ray diffraction (WAXS)

3.2.5.2.1 X-ray pattern and relative crystalllinity

WAXS were obtained with a Rigaku D/MAX-2200V-PC X-ray diffractometer (Rigaku-Denki Co., Tokyo, Japan), with operating conditions as follows: target voltage – 40kV, current – 100mA, aging time – 5 minutes, scan speed – 2.000°/minute, scanning range – 3-35°, step time – 4.5s, divergence slit width – 1.00, scanning slit width – 1.00, receiving slit width – 0.60.

Prior to X-ray studies, the moisture content of the starches were adjusted to approximately 23% by placing the starches in a dessicator containing a saturated solution of potassium sulfate (23-25°C, aw=0.98) for approximately 48 hours. The hydrated samples (0.5g, db) were then tightly packed into an aluminum holder for X-ray analysis.

Crystallinity of the native chickpea starches was quantitatively estimated using the method of Nara and Komiya (1983) by using the Origin computer software (Origin 6.0, Microcal Inc., Northampton, MA, USA). First, a smooth curve that connected peak baselines was computer plotted on the diffratogram where the area above the smooth curve was considered the crystalline portion and the area between the lower portion of the curve and the linear baseline was taken as the amorphous region. The ratio of the upper area to the total diffraction area was calculated as crystallinity according to the following equation:

$$\text{Crystallinity (\%)} = \frac{Ac}{Ac + Aa} \times 100$$

where Ac and Aa are the crystalline and amorphous areas of the X-ray diffractogram, respectively.

3.2.6 Physicochemical Properties

3.2.6.1 Swelling factor (SF)

The swelling factor (SF) measures only intragranular water and hence the true SF at a given temperature. SF of the starches when heated in the temperature range of 50°C – 90°C in excess water was determined according to the method of Tester and Morrison (1990a). Starch samples (50mg, db) were weighed into screw cap tubes; 5mL water was added and heated in a shaking water bath at the specified temperatures for 30 minutes. The tubes were then cooled rapidly to 20°C in an ice water bath and 0.5mL of blue dextran (Pharmacia; MW $2x10^6$, 5mg/mL) was added and the contents mixed by inverting the closed tubes several times. The tubes were centrifuged (1500 x g for 10min) and the absorbance of the supernatant was measured at 620nm. The absorbance of the reference tube that contained no starch was also measured. Three replicate samples were used in this determination. The SF is reported as the ratio of the volume of swollen starch granules to the volume of the dry starch using the equation shown below:

The calculation of the SF of the starches was based on a starch weight corrected to 12% moisture and assumes a density of 1.4g/mL. Free or interstitial-plus-supernatant water (FW) is calculated according to the equation:

$$FW = 5.5\ (A_r/A_s) - 0.5$$

where A_r and A_s are the absorbances of the reference and sample experiments, respectively.

The initial volume of the absorbed intragranular water (V_1) is thus:

$$V_1 = 5.0 - FW$$

And the initial volume of starch (Vo) of weight (W, mg) is:

$$Vo = W/1400$$

Therefore, the volume of the swollen starch granule (V2) is calculated as:

$$V_2 = V_o + V_1$$

And the SF is:

$$SF = V_2/V_0$$

This can also be expressed in a single equation whereby:

$$SF = 1 + \{(7700/W) \times [(A_s - A_r)/A_s]\}$$

3.2.6.2 Extent of amylose leaching (AML)

Starches (20mg, db) in water (10mL) were heated at 50–90°C in volume-calibrated sealed tubes for 30 minutes (tubes were shaken by hand every 5 minutes to re-suspend the starch slurry). The tubes were then cooled to room temperature (23-25°C) and centrifuged at 2000 x g for 10 minutes. The supernatant (1mL) was withdrawn and its amylose content determined using the method of Hoover and Ratnayake (2004).

Amylose leaching was expressed as the percentage of leached amylose per 100g of starch. Three replicate samples were used in the determination.

3.2.6.3 Starch gelatinization

Gelatinization characteristics of native chickpea starches were determined by differential scanning calorimetry (DSC) using a Seiko differential scanning calorimeter (DSC 210, Seiko Instruments Inc., Chiba, Japan), operated under nitrogen and equipped with a thermal station for data analysis and recording. Calibration for temperature and heat flow was performed using indium (mp=156.6°C, ΔH=28.71 J/g, Aldrich Chemical Corp.).

Starch (3.0 mg) was weighed into aluminum DSC pans (ME-00026763100, Seiko Instruments Inc., Chiba, Japan) and 11μL of distilled water was added. The pans were then sealed, weighed, and allowed to stand for 24 hours before analysis. The pans were then heated over a range of 30-130°C, with a heating range of 10°C/minute, using a blank DSC pan as a reference. The gelatinization temperatures that are reported are the onset (T_o), peak (T_p) and conclusion (T_c) temperatures. The enthalpy of gelatinization (ΔH) was estimated by integrating the area between the thermogram and a base line under the peak and was expressed in terms of Joules per gram (J/g) of dry starch. All experiments were repeated in triplicate.

3.2.6.4 Pasting properties

The pasting properties of the starches (7% db, 27g total weight) were determined using a Rapid ViscoTM Analyzer RVA-4 (Newport Scientific Pty. Ltd., Warriewood, NSW, Australia). Starch slurries were equilibrated at 50°C for 1 minute, heated at 6°C/minute to 95°C and then held at 95°C for 5 minutes. After the holding phase, the slurries were cooled at 6°C/minute to 50°C and then held at 50°C for 2 minutes. The spindle speed was 960 rpm for the first 10 seconds (to disperse the sample) and then at 160 rpm for the remainder of the experiment (approximately 23 minutes). The experiments were repeated in duplicate for each chickpea cultivar.

3.2.6.5 Acid hydrolysis

Native chickpea starches (100mg, db) were hydrolyzed in 4mL of 2.2M hydrochloric acid at 35°C for periods ranging from 0-20 days. The starch slurries were vortexed daily to re-suspend the deposited starch granules. Aliquots of the reaction mixture were removed at specific time intervals, neutralized with 2.2M NaOH, and centrifuged (1000 x g for 10 minutes). The extent of hydrolysis was determined by expressing the solubilized carbohydrates (Jane and Robyt, 1984) as a percentage of the initial starch using the method of Bruner (1964).

3.2.6.5.1 Bruner method for reducing sugar determination

Materials:

3,5-dinitrosalicyclic acid (DNS, 20g) was dissolved in 700mL of NaOH (1M) by constant stirring overnight. The volume was adjusted with distilled water to a final volume of 1 liter and the solution was filtered through a medium-porosity glass filter.

Method:

The DNS solution (2mL) was cooled in an ice bath for 5 minutes prior to adding the aliquot (1mL) of the acid-hydrolyzed supernatant and 1mL of distilled water. The mixture was then heated in a boiling water bath for exactly 5 minutes and cooled on ice for 10 minutes. After cooling, the final volume was adjusted to 12mL by adding distilled water. The absorbance was monitored at 540nm for starches subjected to acid for less than 24 hours, and at 590nm for starches subjected to acid for 24 or more hours. Standard curves were constructed with known glucose concentrations at both 540nm and 590nm (see appendix). The extent of hydrolysis was expressed as the amount of glucose released as a percentage of the initial starch according to the flowing equation:

$$\text{Hydrolysis (\%)} = \frac{\text{Reducing sugar released as glucose (g)} \times 0.90 \times 100}{\text{Initial starch weight (g)}}$$

3.2.6.6 Enzyme digestibility

The *in vitro* digestion of starch was determined using the Englyst (1992) method with some minor modifications.

Materials:

Pancreatic α-amylase plus amyloglucosidase solution: Porcine pancreatin (0.45g, Sigma P7545) was added to 4mL of water and stirred using a magnetic stir bar for 5 minutes. The mixture was centrifuged (1500 x g) for 10 minutes. The cloudy supernatant was decanted and an aliquot (2.7mL) was placed in a beaker. Amyloglucosidase (0.32mL, Megazyme E-AMGDF) was then diluted to a final volume of 0.4mL with distilled water and an aliquot (0.3mL) of this solution added to the beaker containing the pancreatin. Invertase (2mg in 0.2mL , Sigma I4504) was also added to the enzyme solution.

Buffer concentrate: Potassium dihydrogen orthophosphate (136g potassium phosphate monobasic), 42g of sodium hydroxide and 30g of 4-hydroxybenzoic acid was dissolved in 900mL of distilled water. The pH was adjusted to7.4 with either 2.0M HCl or 2.0M NaOH and the solution was diluted to 1 litre and mixed well until dissolved.

Glucose oxidase-peroxidase reagent (GOPOD): Buffer concentrate (50mL) was diluted to 1 liter. The vial containing the freeze-dried glucose oxidase-peroxidase-aminoantipyrine mixture was then quantitatively transferred to the 1 liter volumetric flask containing the diluted buffer. The resultant mixture contained: glucose oxidase > 12 000 U/L; peroxidase > 650 U/L; 4-aminoantipyrine 0.4mM.

Method:

Starches from all four chickpea cultivars were accurately weighed (100mg, db) in glass test tubes and 15 glass beads (4mm in diameter) were added to each tube. 2mL of 0.05M HCl/guar gum solution (4.1mL conc. HCl/; 5 mg/mL guar gum) was added to each tube and the contents mixed by vortexing. Pepsin (10mg, Sigma P7125) was then added and the tubes were incubated at 37°C with constant shaking for 30 minutes. After incubation, 4mL of 0.5M sodium acetate buffer (pH 5.2) with 20mM $CaCl_2$ was added to each tube, vortexed, and placed horizontally in a 37°C shaking water bath for 5 minutes before adding 1mL of the enzyme solution. Aliquots (100 μL) were removed at 20 and 120 minutes of incubation and mixed with 1 mL of 50% ethanol, and then diluted by adding 3mL of distilled water. The solutions were then mixed by vortexing and centrifuged at 1000 x g for 10 min. The GOPOD solution (3mL) was added to 100μL of the diluted supernatant in a test tube which was then incubated for 20 minutes at 50°C. The absorbance of the solution was measured spectrophotometrically at 510 nm against a reagent blank constructed by replacing 100 μL of distilled water with the diluted enzyme digested starch solution. The amount of glucose produced was determined from a standard curve of known glucose concentrations prepared by pipetting 25, 50, 75 and 100 μL of standard glucose solution (Megazyme, 1 mg/mL) into test tubes to which distilled water was added to bring the final volume to 100 μL. The percentage of digestible starch was calculated according to the following equation:

% Digestible Starch = G x (5/0.1) x (4.1/0.1) x (1/1000) x (100/DM) x (162/180)

where: G - mass of glucose (μg) calculated from the standard curve

5/0.1 - volume correction for aliquot removed at different hydrolysis times

4.1/0.1 - volume correction for GOPOD step

1/1000 - conversion from μg to mg of glucose

DM - dry mass of sample (mg) = "as is" sample mass – (100- % moisture)/100

100/DM - factor to express digestible starch as a % of dry sample mass

162/180 - factor to convert free glucose (as it was measured) into anhydro-glucose, as it occurs in starch.

The percentage of rapidly digestible starch (RDS) and slowly digestible starch (SDS) was correlated with the 20 and120 minute intervals of digestion, respectively, while the percentage of resistant starch (RS) was taken as the amount of starch remaining undigested after 120 minutes.

3.2.6.7 Turbidity measurements

The turbidity of the chickpea starch samples was determined using the method of Perera and Hoover (1999). A 2% aqueous suspension (pH 7.0) of starch was heated for 1 hour and 95°C and then stored at 4°C for 24 hours (to increase nucleation), followed by 1 to 15 days at 40°C. The development of turbidity at specific time intervals was estimated by measuring the absorbance at 640nm against a water blank.

3.2.7 Statistical analysis

All determinations were replicated thrice and the mean values and standard deviations were reported. Analysis of variance (one way ANOVA) was performed by Tukey's HSD test ($P<0.05$) using the Statistical Software SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

Chapter 4: Results and Discussion

4.1 Chemical composition

The data on yield and composition of the four chickpea cultivars are presented in **Table 4-1.** The yield of isolated of pure starch was of 32.01 to 36.9%, decreasing in descending order: CDC Flip 97-133c > CDC 418-59 > CDC Xena > CDC ICC 12512-9. The starch yield from chickpea was in the 12-46% range reported for starches from pulses and other chickpea cultivars (Chavan et al., 1999; Chung et al., 2008a; Grelda et al., 1997; Hoover and Ratnayake, 2002; Hoover and Sosulski, 1991; Hoover et al., 1997; Hoover, Swamidas and Vasanthan, 1993; Jayakody et al., 2007; Ratnayake et al., 2001; Srisuma et al., 1994; Zhou, Hoover and Liu, 2004).

The purity of the chickpea starches was judged on the basis of the low nitrogen (0.03-0.06%) content (**Table 4-1**) and the absence of any adhering protein on the granule surface (**Figure 4-1**). The nitrogen content of the chickpea cultivars was comparable to those reported for other pulse starches (0.02 to 0.11% (**Table 2-2**)).

The total lipid content of starches from the chickpea cultivars ranged from 0.20% to 0.50% and followed the order: CDC 12512-9~CDC 418-59 > CDC Flip 97-133c > CDC Xena (**Table 4-1**). The above values were within the range (0.07 to 0.82%) reported for starches from pulses and other chickpea cultivars (Betancur-Ancona et al., 2002a; Betancur-Ancona et al., 2002b; Chavan et al., 1999; Chung et al., 2008a; Grelda et al., 1997; Hoover and Manuel, 1995; Hoover and Ratnayake, 2002; Hoover, Swamidas and Vasanthan, 1997; Hoover et

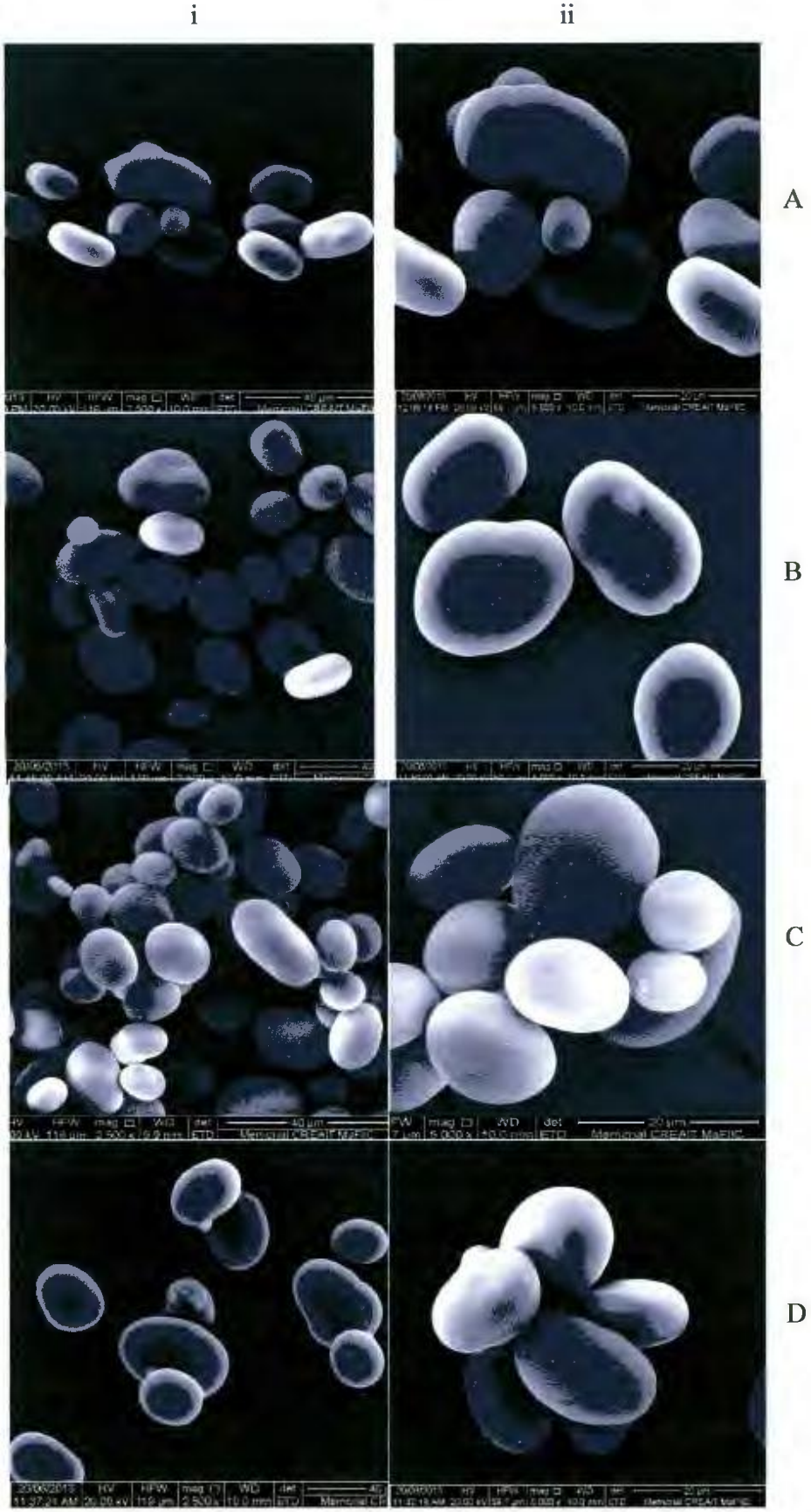
Table 4-1: Chemical composition (%)[1] of native chickpea starches

Characteristics	Chickpea Cultivar			
	CDC Xena	CDC Flip 97-133c	CDC 418-59	CDC ICC 12512-9
Yield (% of initial seeds)	34.4 ± 0.7^{a,b}	36.8 ± 2.9^{a,b}	35.9 ± 1.9^{a,b}	32.0 ± 0.7^{a}
Moisture	11.45 ± 0.09^{a}	10.57 ± 0.18^{b,c}	10.45 ± 0.11^{c}	8.78 ± 0.21^{d}
Nitrogen	0.03 ± 0.01^{a}	0.05 ± 0.02^{a}	0.04 ± 0.01^{a}	0.06 ± 0.01^{a}
Lipid				
Chloroform-methanol[2]	0.08 ± 0.02^{a}	0.04 ± 0.02^{a,b}	0.13 ± 0.04^{a,c}	0.04 ± 0.02^{a,b,c}
n-propanol-water[3]	0.21 ± 0.08^{a,b}	0.36 ± 0.08^{a,b}	0.37 ± 0.01^{a,b}	0.46 ± 0.5^{b}
Amylose content				
Apparent amylose[4]	30.78 ± 0.17^{c}	33.81 ± 0.17^{a}	33.71 ± 0.46^{a}	32.60 ± 0.17^{b}
Total amylose[5]	33.86 ± 0.46^{c}	40.22 ± 0.35^{a}	38.40 ± 0.46^{b}	37.49 ± 0.46^{b}
Amylose complexed with native lipid[6]	9.08 ± 1.24^{c}	15.93 ± 0.73^{a}	12.21 ± 1.05^{b}	13.05 ± 1.07^{b}
Starch damage[7]	ND	ND	ND	ND

[1]All data represent the mean of triplicates. Values followed by the same superscript in each row are not significantly different ($P<0.05$) by Tukey's HSD test.
[2]Lipids extracted by chloroform-methanol 2:1 (v/v) at 25°C (mainly unbound lipids).
[3]Lipids extracted by hot n-propanol-water 3:1 (v/v) from the residue left after chloroform-methanol extraction (mainly bound lipids).
[4]Apparent amylose determined by iodine binding without removal of free and bound lipids.
[5]Total amylose determined by iodine binding after removal of free and bound lipids.
[6]$\frac{\text{Total amylose} - \text{Apparent amylose}}{\text{Total amylose}} \times 100$
[7]Not detected.

Figure 4-1: Morphology of starch granules using SEM at i) 2500x and ii) 5000x magnification for four chickpea cultivars:

A) CDC ICC 12512-9, B) CDC 418-59, C) CDC Xena, D) CDC Flip 97-133c


i
ii
A
B
C
D

al., 1993; Huang et al., 2007a; Huang et al., 2007b; Ratnayake et al., 2001; Singh, Sandhu and Kaur, 2004). The bound lipid content (**Table 4-1**) followed the order: CDC ICC 12512-9 (0.46%) > CDC 418-59 (0.37%) ~ CDC Flip 97–133c (0.36%) > CDC Xena (0.21%). This was in the range of 0.08 to 0.8% of bound lipid reported for other pulse starches (**Table 2-1**). However, there are no reports in the literature for the bound lipid content in starches from other chickpea cultivars. The surface lipid content (0.04-0.13%) of the chickpea cultivars followed the order: CDC ICC 12512-9 ~ CDC Flip 97-133c > CDC Xena > CDC 418.59. The above values were within the range (0.10-0.20%) reported for other pulse starches (**Table 2-1**).

The total amylose content (**Table 4-1**) of the chickpea starches followed the order: CDC Flip 97-133c (40.22%) > CDC 418-59 (38.40%) ~ CDC ICC 12512-9 (37.49%) > CDC Xena (33.86%). The above values were lower than those reported for mung bean (45.3%, Hoover et al., 1997), field pea (48.8 – 49.6%, Ratnayake et al., 2001), smooth pea (52.6 – 57.0%, Czuchajowska et al., 1998) and chickpea (var suratato 77; 46.5%; Grelda et al., 1997) starches, but was higher than those reported for starches from other cultivars of chickpea (23.0 – 34.3%; El Tinay et al., 1983; Huang et al., 2007b; Singh, Sandhu and Kaur, 2004; Hoover and Ratnayake, 2002). A comparison of the apparent and total amylose content (**Table 4-1**) showed that 9.08, 15.93, 12.21 and 13.05% of the total amylose was complexed by native lipids in CDC Xena, CDC Flip 97-133c, CDC 418-59 and CDC ICC 12512-9, respectively. The corresponding values for starches from other pulses and other chickpea cultivars have been shown to be in the

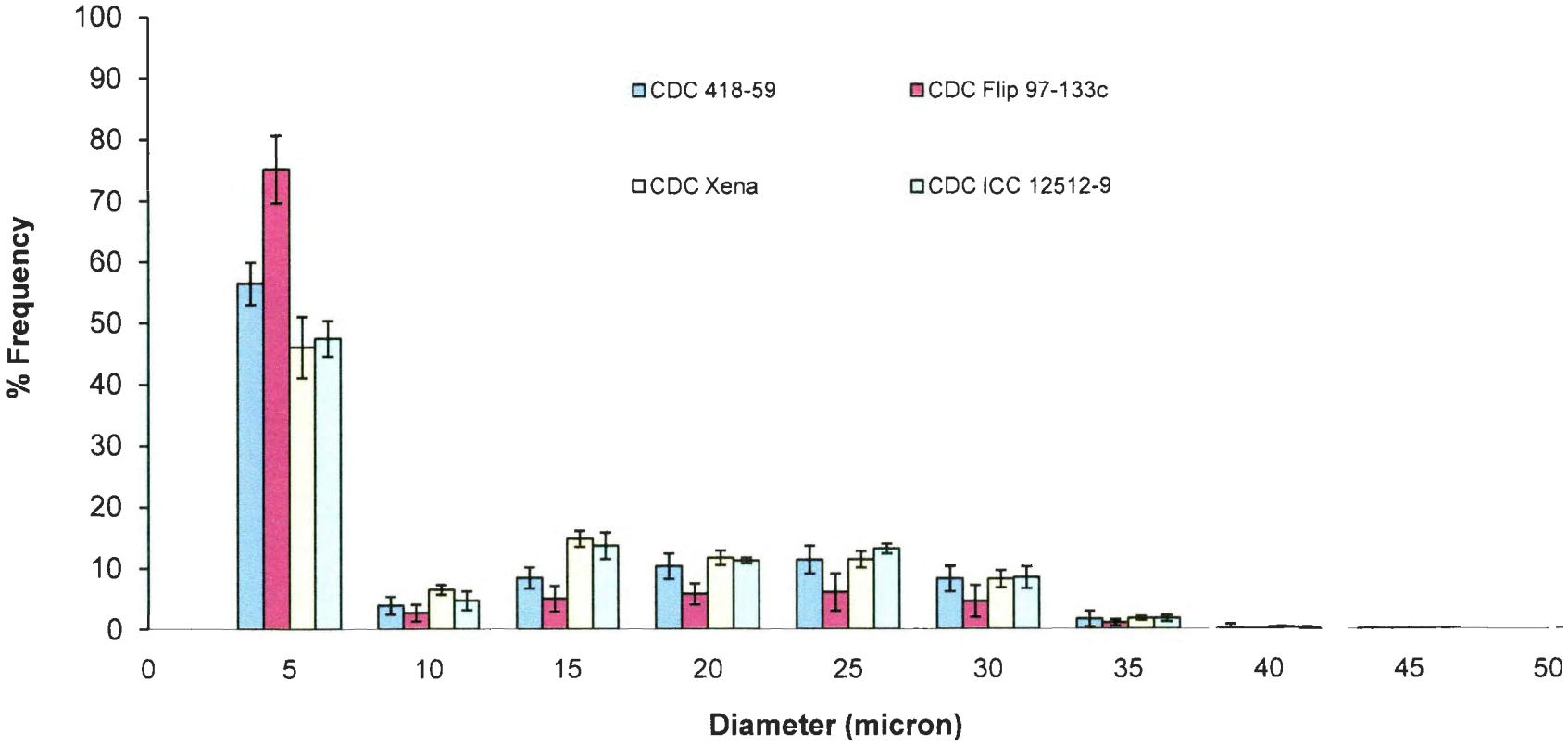
range 6.0-14.9% and 9-10%, respectively (Hoover and Ratnayake, 2002; Ratnayake et al., 2001).

4.2 Granule morphology and particle size distribution

Starch granules of all four chickpea cultivars ranged from large oval shaped to small spherical granules (**Figure 4-1**). The granule surface appeared to be smooth and showed no evidence of fissures (**Figure 4-1**) when viewed under the scanning electron microscope (SEM).

The particle size distribution of the chickpea starch granules plotted as frequency (%) of distribution versus granule diameter (microns) is presented in **Figure 4-2**. The results showed that CDC Flip 97-133c had the highest frequency of granules with diameters up to 5μ, followed by CDC 418-59, CDC ICC 12512-9 and CDC Xena, respectively. In the 10 to 15 μ diameter range, CDC Xena exhibited the maximum percentage granule distribution followed by CDC ICC 12512-9, CDC 418-59 and CDC Flip 97-133c, respectively. In the 20 to 30 μ diameter range, CDC Flip 97-133c exhibited the lowest distribution frequency, whereas the 20 to 30 μ distribution was comparable in the other cultivars. Microscopy observations (Singh, Sandhu and Kaur, 2004; Hoover and Ratnayake, 2002) have shown that starch granule size in chickpea starches are generally in the range 6-31 μm, which is in fairly close agreement with this study.

Figure 4-2: Particle size distribution of four chickpea cultivars (particle diameter plotted against the frequency)


100
90
80
70
60
50
40
30
20
10
0
% Frequency
CDC 418-59
CDC Flip 97-133c
CDC Xena
CDC ICC 12512-9
0
5
10
15
20
25
30
35
40
45
50
Diameter (micron)

4.3 Amylopectin chain length distribution

The normalized branch chain length distribution and the average chain length ($\overline{CL}$) of the debranched amylopectins of the four chickpea cultivars were nearly similar (**Table 4-2**). The percentage chain distribution in all four starches followed the order: DP 13-24 > DP 6-12 > DP 25 – 36 > DP 37-54. This was similar to that reported for field pea (Ratnayake et al., 2001), kidney bean (Yoshida et al., 2003) and for another unspecified chickpea cultivar (Huang et al., 2007b). The $\overline{CL}$ in most pulse starches have been shown to be in the 17.3-25.0 range (Biliaderis et al., 1981; Chung et al., 2008a,b; Jayakody et al., 2007; Ratnayake et al, 2001; Yoshida et al., 2003; Yoshimoto et al., 2001). However, the corresponding values for the chickpea starches (17.79-17.89) were lower.

According to the revised cluster model of amylopectin (Hanashiro et al., 1996; Hizukuri, 1986), short chains with DP 6-24 comprise A and B1 chains. These chains are arranged in double helices, and are mainly located in the crystalline domains of starch granules. Therefore, similarity in distribution of DP 6-24 chains among the four chickpea starches **(Table 4-2)** suggests identical crystalline structures.

4.4 X-ray pattern and relative crystallinity

The four chickpea starches exhibited the characteristic C-type X-ray pattern of pulse starches (Chavan et al., 1999; Gernat et al., 1990; Hoover and Ratnayake, 2000; Hoover and Sosulski, 1985; Huang et al. 2007b; Jayakody et al., 2007). In the chickpea

Table 4-2: Amylopectin branch chain length distribution and average chain length ($\overline{CL}$) of chickpea starches

	% Distribution $(dP_n)^2$				
Chickpea Cultivar	6-12	13-24	25-36	37-54	$\overline{CL}$ [3]
CDC Xena	29.28 ± 1.04[a]	52.84 ± 0.66[a]	13.97 ± 0.46[a]	3.90 ± 0.07[a]	17.89 ± 0.12[a]
CDC Flip 97-133c	30.00 ± 1.26[a]	51.89 ± 0.69[a]	14.28 ± 0.54[a]	3.83 ± 0.11[a]	17.84 ± 0.19[a]
CDC 418-59	29.45 ± 1.21[a]	52.70 ± 0.55[a]	14.01 ± 0.44[a]	3.84 ± 0.36[a]	17.85 ± 0.25[a]
CDC ICC 12512-9	29.74 ± 1.24[a]	52.73 ± 0.67[a]	13.82 ± 0.38[a]	3.71 ± 0.23[a]	17.79 ± 0.20[a]

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

[2]Indicates degree of polymerization

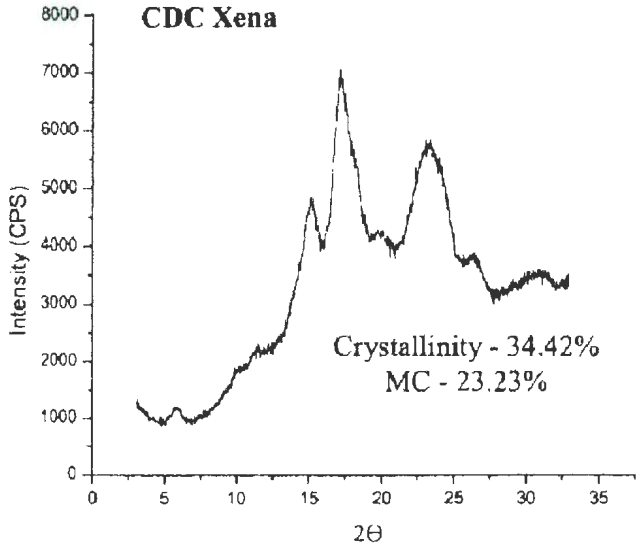
[3]Average chain length ($\overline{CL}$) calculated by (dP_n x peak area)/(peak $area_n$)

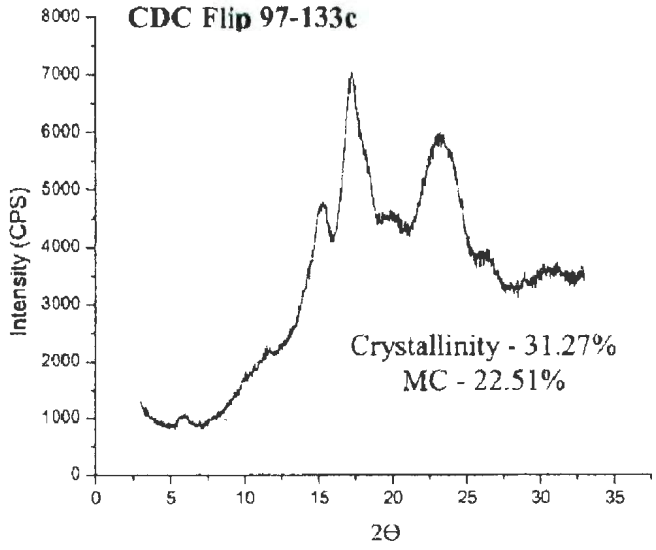
starches studied, the 'C' pattern was characterized by a weak peak at $2\theta = 5.4^{o}$ (characteristic of 'B' polymorphs) and strong peaks at 17.5 and 23^{o} 2θ (**Figure 4-3**). The intensity of the peak at 5.4^{o} 2θ was identical in all four starches. This suggests that the chickpea starches have the same proportion of 'B' unit cells. The relative crystallinity followed the order: CDC ICC 12512-9 > CDC Xena > CDC 418-59 > CDC Flip 97-133c. These differences, although small, were significant ($P < 0.05$). This was rather surprising, since the above starches did not differ in amylopectin structure (**Table 4-2**). This suggests that the crystallites in these starches are oriented differently. Although the relative crystallinity was within the range (17.0 – 34.0%) reported for other pulse starches (Davydova et al., 1995; Hoover and Ratnayake, 2002; Ratnayake et al., 2001; Xu et al., 2013), a reliable comparison of relative crystallinity cannot be made, since the X-ray data reported in the literature have been obtained at different moisture contents.

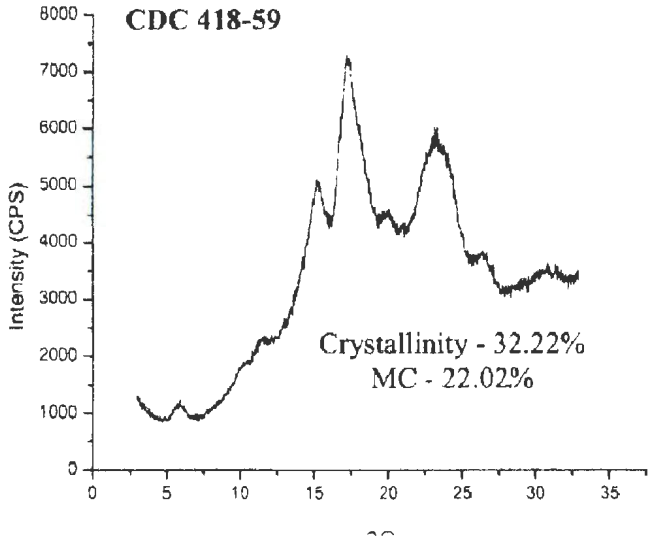
4.5 Swelling factor (SF)

The SF of chickpea starches in the temperature range of 50-90°C is presented in **Table 4-3**, with the pattern of swelling shown in **Figure 4-4**. The SF of all starches increased dramatically in the temperature range of 65-70°C. A similar trend has also been reported to occur in other pulse starches (Chavan et al., 1999; Hoover and Manuel, 1995; Ratnayake et al., 2001). In the temperature range of 50-65°C, CDC Flip 97-133c exhibited the highest SF. Differences in SF among the other starches were marginal. At

Figure 4-3: X-ray diffraction pattern of four cultivars of native chickpea starches showing relative crystallinity (%).


CDC Xena
Intensity (CPS)
2Θ
Crystallinity - 34.42%
MC - 23.23%


CDC Flip 97-133c
Intensity (CPS)
2Θ
Crystallinity - 31.27%
MC - 22.51%


CDC 418-59
Intensity (CPS)
Crystallinity - 32.22%
MC - 22.02%

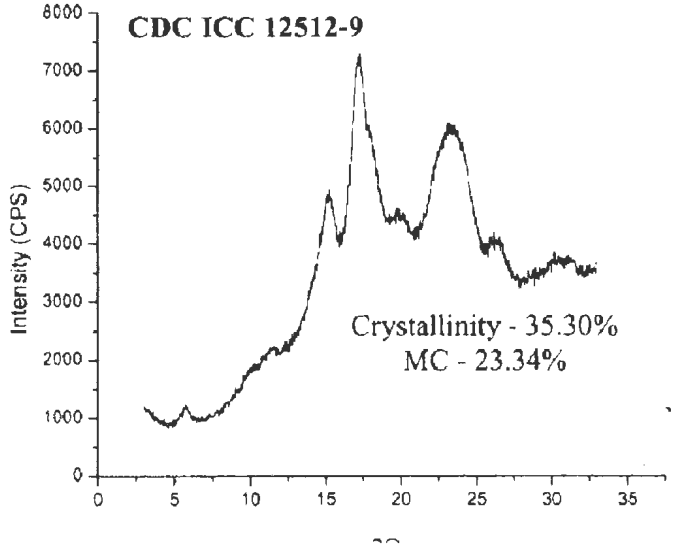

CDC ICC 12512-9
Intensity (CPS)
Crystallinity - 35.30%
MC - 23.34%

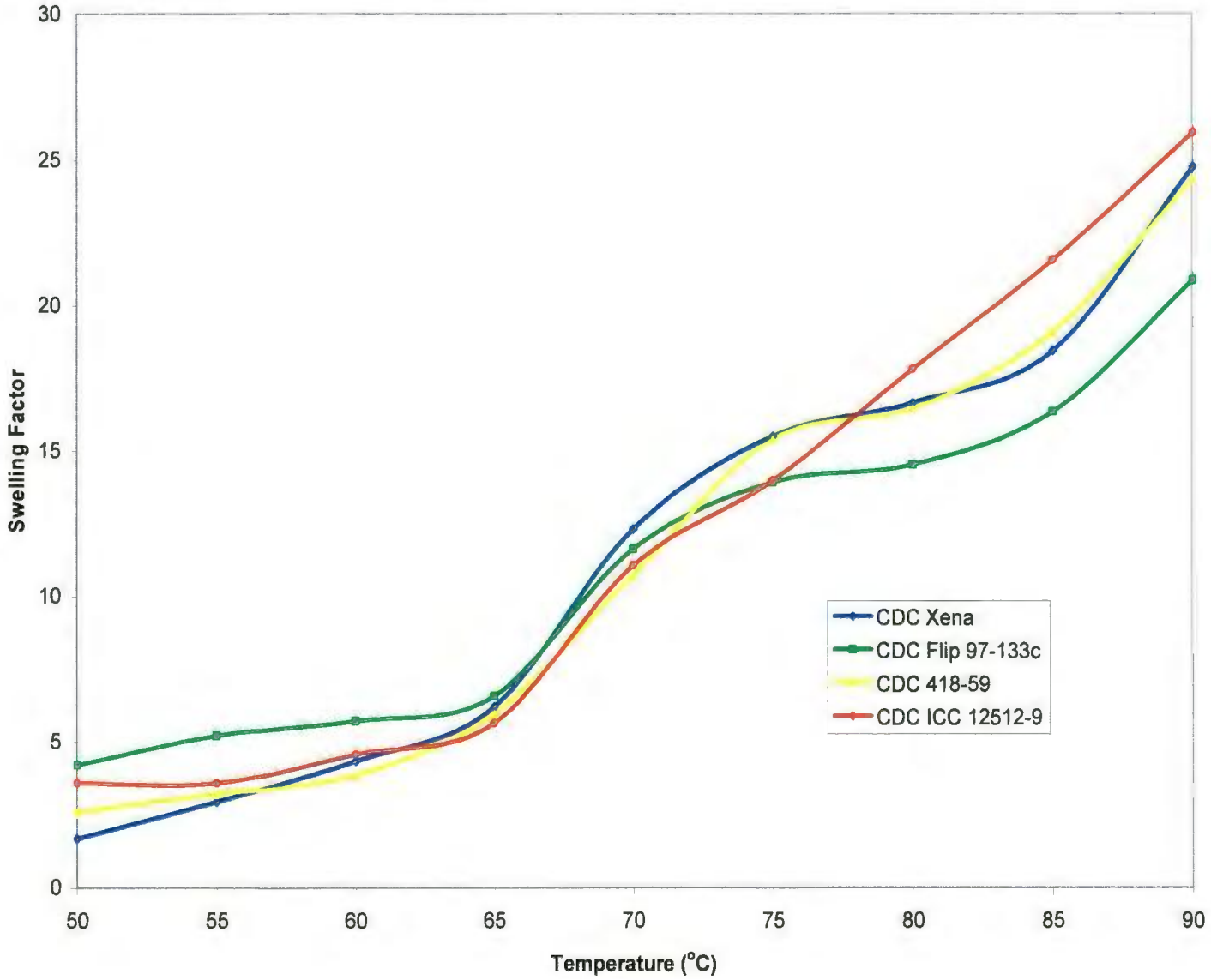
Table 4-3: Swelling factor (SF) and amylose leaching (AML) of chickpea starches

Chickpea cultivar	Temperature (°C)								
	50	55	60	65	70	75	80	85	90
CDC Xena									
SF	1.68±0.39[a]	2.93±0.38[a]	4.33±0.43[a]	6.19±0.42[a,b]	12.29±0.34[a]	15.47±0.49[a]	16.65±0.18[a]	18.42±0.54[a]	24.75±0.49[a]
AML	--[2]	--[2]	--[2]	--[2]	9.36±0.30[w,x]	21.18±0.30[w]	28.56±0.17[w]	30.17±0.35[w]	36.13±0.35[w]
CDC Flip97-133c									
SF	4.20±0.43[b]	5.20±0.37[b]	5.70±0.21[b]	6.55±0.21[b]	11.61±0.34[a,b]	13.89±0.32[b]	14.50±0.50[b]	16.33±0.49[b]	20.89±0.30[b]
AML	--[2]	--[2]	--[2]	--[2]	10.07±0.46[w]	18.86±0.35[x]	25.83±0.35[x]	29.16±0.17[x]	35.42±0.30[w]
CDC 418-59									
SF	2.55±0.35[a,c]	3.19±0.22[a]	3.83±0.22[a]	5.94±0.37[a,b]	10.69±0.40[c]	15.37±0.37[a]	16.43±0.49[a]	19.04±0.31[a]	24.33±0.28[a]
AML	--[2]	--[2]	--[2]	--[2]	8.66±0.17[x]	15.93±0.17[y]	24.31±0.35[y]	28.86±0.17[x]	32.90±0.46[x]
CDC lCC12512-9	3.57±0.44[b,c]	3.58±0.22[a]	4.56±0.38[a]	5.63±0.45[a]	11.04±0.20[b,c]	13.95±0.33[b]	17.80±0.00[c]	21.54±0.17[c]	25.92±0.42[c]
SF	--[2]	--[2]	--[2]	--[2]	8.61±0.46[x]	15.22±0.17[z]	25.22±0.17[x]	26.84±0.17[y]	32.80±0.46[x]
AML									

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

[2] - Indicates not detected.

Figure 4-4: Swelling factor pattern of four native chickpea starches


30
25
20
15
10
5
0
Swelling Factor
50
55
60
65
70
75
80
85
90
Temperature (°C)
CDC Xena
CDC Flip 97-133c
CDC 418-59
CDC ICC 12512-9

70 and 75°C, SF was higher for CDC Xena, although differences in SF order could not be clearly discerned in the other three starches. In the temperature range of 80-90°C, SF followed the order: CDC ICC 12512-9 > CDC Xena > CDC 418-59 > CDC Flip 97-133c.

According to **Figure 4-4**, the SF of CDC Xena and CDC Flip 97-133c increased linearly with increasing temperature from 50 to 65°C, whereas the CDC ICC 12512-9 cultivar showed a linear relationship with temperature increase from 75 to 90°C. Furthermore, the SF of CDC ICC 12512-9 showed primarily a two stage process compared to the multistep swelling of starch from other cultivars.

SF has been shown to be influenced by the amount of lipid complexed amylose chains (Hoover and Manuel, 1996; Maningat and Juliano, 1980; Tester and Morrison, 1990a) and amylopectin molecular structure (Tester et al., 1993). It is unlikely, that the difference in SF among the chickpea starches is influenced by the former, since on this basis, SF should have followed the order: CDC Xena > CDC 418-59 ~ CDC ICC 12512-9 > CDC Flip 97-133c. Differences in SF cannot be explained on the basis of amylopectin structure, since there was no significant variation in amylopectin branch chain length distribution among the starches (**Table 4-2**).

This suggests that the main causative factor influencing differences in SF among the chickpea starches may have been due to differences in their crystallinity **(Figure 4-3)**. This seems plausible since differences in SF among the starches in the temperature range 80-90°C, followed the crystallinity order: CDC ICC 12512-9 > CDC Xena > CDC 418-59 > CDC Flip 97-133c. The higher crystallinity of CDC ICC 12512-9 would prevent

rapid granular swelling at lower temperatures, but would permit granules to swell without disintegration at higher temperatures. This would then explain the lower SF at temperatures below 65°C, and the higher SF at temperatures in the range 80-90°C (**Table 4-3**) for CDC ICC 12512-9.

The SF of the chickpea starches was much lower than those of other chickpea cultivars reported in the literature (Hoover and Ratnayake, 2002). For instance, the SF at 60°C for starches from chickpea cultivars Desiray and Yuma were 18.2 and 15.0, respectively (Hoover and Ratnayake, 2002). Chung et al. (2008a) reported that SF for three chickpea cultivars (Myles, Flip 97-101c and 97-Indian2-11) were in the range of 5.1-9.4, 13.3-16.1, 19.1-23.1, and 22.6-23.4 at 60, 70, 80, and 90°C, respectively, where the SF were lower as well, with the exception of 90°C, where the SF was minimally higher (20.89-25.92) in all cultivars except for Flip 97-133c.

4.6 Amylose leaching (AML)

In all starches, AML was not detected at temperatures below 70°C (**Table 4-3**). The extent of AML in the 75-90°C temperature range followed the order: CDC Xena > CDC Flip 97-133c > CDC 418-59 > CDC 12512-9. The results suggest that AML in the chickpea starches is influenced by the interplay between differences in: 1) total amylose content (CDC Flip 97-133c > CDC 418-59 ~ CDC ICC 12512-9 > CDC Xena), 2) amount of lipid complexed amylose chains (CDC Flip 97-133c> CDC ICC 12512-9 ~ CDC 418-59 > CDC Xena) and 3) extent of interaction between amylose-amylose and/or

amylose-amylopectin chains. This seems plausible, since if amylose content was the sole factor influencing AML, then CDC Flip 97-133c and CDC Xena should exhibit the highest and lowest extent of AML among the four starches, respectively. Thus, the difference in AML between CDC Xena and CDC Flip 97-133c may be attributed to the lower content of lipid complexed amylose chains (**Table 4-1**) and/or to weaker interaction between amylose-amylose and/or amylose-amylopectin chains in the former. The absence of any significant difference in total amylose content and lipid complexed amylose chains between CDC ICC 12512-9 and CDC 418-59 starches (**Table 4-1**), could explain their marginal difference in AML (**Table 4-3**). AML in starches from pulses and other chickpea cultivars have been observed to occur even at 60°C (**Table 2-6**).

4.7 Gelatinization characteristics

The gelatinization transition temperatures (T_o (onset), T_p (midpoint), T_c (conclusion)), gelatinization temperature range ($T_c - T_o$) and the enthalpy of gelatinization (ΔH) are presented in **Table 4-4**. There was no significant difference in T_o and T_p among the four starches. However, T_c and ΔH followed the order: CDC Xena > CDC Flip 97-133c > CDC ICC 12512-9 > CDC 418-59 (**Table 4-4**).

T_o, T_p and T_c may be influenced by amylose content (Protserov et al., 2000; Stevenson, Domoto and Jane, 2006; Visser et al., 1997) distribution of amylopectin chains (Stevenson, Domoto and Jane, 2006; Vandeputte et al., 2003; Noda et al., 1998), and lipid complexed amylose chains (Hoover and Ratnayake, 2002; Jayakody et al.,

Table 4-4 Gelatinization characteristics of chickpea starches[1]

	Gelatinization parameters				
Chickpea cultivar	T_o (°C)[2]	T_p (°C)[2]	T_c (°C)[2]	$T_c - T_o$ (°C)[3]	ΔH (J/g)
CDC Xena	59.83 ± 0.08[a]	65.07 ± 0.04[a]	79.28 ± 0.31[a]	19.45 ± 0.35	13.01 ± 1.50[a]
CDC Flip 97-133c	58.65 ± 0.35[b]	63.29 ± 0.60[b]	79.00 ± 0.33[b]	20.35 ± 0.49	12.58 ± 1.72[b]
CDC 418-59	59.01 ± 0.51[b]	64.67 ± 0.38[a]	77.47 ± 0.38[c]	18.47 ± 0.88	11.16 ± 2.31[c]
CDC ICC 12512-9	59.48 ± 0.34[a,b]	65.51 ± 0.27[a]	78.60 ± 0.08[d]	19.12 ± 0.42	11.98 ± 0.26[d]

[1]All data reported on dry basis and represent the mean of three replicates. Values followed by the same superscript in each column are not significantly different ($P < 0.05$) by Tukey's HSD test.

[2]T_o, T_p, T_c indicate the onset, peak and conclusion temperature of gelatinization, respectively.

[3]T_c-T_o represents the gelatinization temperature range.

[4]ΔH represents the enthalpy of gelatinization.

2005; Vandeputte et al., 2003). T_o has been postulated to represent the melting of the weakest crystallites (Larsson and Eliasson, 1991; Nakazawa and Wang, 2003; Wang, Powell and Oates, 1997), whereas, T_c represents melting of high stability crystallites (Jacobs et al., 1998). In this case, low T_o, T_p, T_c and ΔH represent an abundance of short amylopectin chains in the starch (Noda et al., 1998), whereas higher gelatinization temperatures are an indication of more perfect crystals (Sasaki and Matsuki, 1998), longer chains in the crystals, or larger crystal size (Huang et al, 2007b; Matveev et al, 2001)

However, there is still no consensus with regard to what ΔH represents during the gelatinization process. ΔH has been suggested to reflect the following: 1) melting of double helices (Cooke and Gidley, 1992), 2) overall crystallinity (quality and amount of crystallites) of amylopectin (Tester and Morrison, 1990a) and 3) melting of imperfect amylopectin-based crystals with potential contribution from both crystal packing and helix melting enthalpies (Lopez-Rubio, et al., 2008).

Difference in T_c and ΔH among the chickpea starches cannot be explained on the basis of amylopectin chain length distribution (nearly identical in all starches (**Table 4-2**)) or on the amount of amylose-lipid complexed chains (on this basis, CDC Xena (**Table 4-1**) should have exhibited the lowest T_c and ΔH among the four starches). Thus, the difference in T_c and ΔH among the chickpea starches, suggests that crystallites in CDC Xena are probably larger or more numerous or are of higher stability (this could occur if double helices that form the crystallites are tightly packed). Protserov et al. (2000) showed that an increase in amylose content in starch granules leads to an increase in the

extent of crystalline defects and, correspondingly, to a decrease in gelatinization transition temperatures and ΔH. Thus, it is likely, that the low amylose content (**Table 4-1**) of CDC Xena may have also been a factor influencing its higher T_c and ΔH (**Table 4-4**). The gelatinization temperature range and ΔH of the chickpea starches were within the range reported for the pulse starches and for other cultivars of chickpea (50-80°C, 9.7 – 18.1 J/g; Hoover and Ratnayake, 2002; Huang et al., 2007b; Ratnayake et al., 2001; Singh, Sandhu and Kaur, 2004).

4.8 Pasting characteristics

The pasting characteristics of the chickpea starches are presented in **Table 4-5**. The peak viscosity, breakdown viscosity and setback followed the order: CDC Xena > CDC Flip 97-133c > CDC 418-59 > CDC ICC 12512-9, whereas, the time taken to reach peak viscosity and the pasting temperature followed the order: CDC ICC 12512-9 > CDC 418-59 > CDC Flip 97-133c > CDC Xena.

Pasting properties of starches have been shown to be influenced by molecular structure (amylopectin chain length distribution, molecular weight of amylose and amylopectin, crystallinity) and composition (amylose/amylopectin ratio, amount of lipid complexed amylose chains) (Han and Hamaker, 2001; Lan et al., 2008; Shibanuma, Takeda and Hizukuri, 1996; Zeng et al., 1997). As shown earlier (**Table 4-2**), the chickpea starches did not significantly differ from each other with regard to amylopectin

Table 4-5: Pasting properties of chickpea starches[1]

Chickpea Cultivar	RVA Parameters						
	Peak viscosity (Cp)[2]	Trough viscosity (Cp)[2]	Viscosity breakdown[3] (Cp)[2]	Final viscosity (Cp)[2]	Setback[4] (Cp)[2]	Peak time (min)	Pasting temp (°C)
CDC Xena	4174 ± 11[a]	2867 ± 70[a]	1308 ± 59[a]	7147 ± 66[a]	4281 ± 4.0[a]	6.67 ± 0.09[d]	68.48 ± 0.32[c]
CDC Flip 97-133c	3523 ± 26[b,c]	2842 ± 67[a]	681 ± 93[b,c]	6685 ± 98[b,c]	3843 ± 165[b,c]	8.07 ± 0.0[c]	67.98 ± 0.46[c]
CDC 418-59	3479 ± 11[c]	3011 ± 4[a]	469 ± 15[c]	6509 ± 33[c]	3498 ± 30[c]	8.40 ± 0.0[b]	69.85 ± 0.07[a,b]
CDC ICC 12512-9	3223 ± 16[d]	2829 ± 45[a]	394 ± 30[d]	5939 ± 40[d]	3110 ± 86[d]	8.73 ± 0.09[a]	70.48 ± 0.32[a]

[1]At 7% w/w starch suspension. Values followed by the same letter in the same column are not significantly different ($P<0.05$) by Tukey's HSD test. All data represent the mean of three replicates.

[2]Centipoise

[3]Peak viscosity – trough viscosity

[4]Final viscosity – trough viscosity

chain length distribution. This suggests that the high peak viscosity of CDC Xena reflects its higher amylopectin content (**Table 4-1**), lower content of lipid complexed amylose chains (**Table 4-1**), and higher extent of amylose leaching (**Table 4-1**). The above factors would enable granules of CDC Xena to swell rapidly and reach peak viscosity without much granule disintegration. The more pronounced viscosity breakdown (during the holding cycle) and setback (during the cooling cycle) in CDC Xena (**Table 4-5**), reflects higher granular swelling (makes granules more susceptible to shear) and rapid aggregation of leached amylose chains (due to more extensive amylose leaching (**Table 4-3**)), respectively.

The difference in RVA parameters among the other three cultivars is probably influenced by differences in crystallinity (CDC ICC 12512-9 > CDC 418-9 > CDC Flip 97-133c), since differences in amylose content and lipid complexed amylose chains was marginal among the above starches (**Table 4-1**). The higher crystallinity of CDC ICC 12512-9 (**Figure 4-3**) would explain its lower peak viscosity, higher thermal stability (low breakdown viscosity), and the longer time taken to reach peak viscosity (**Table 4-5**).

No comparison is possible with the pasting properties of starches from other pulses and other chickpea cultivars due to differences in instrumentation (Brabender Viscoamylogram versus RVA) and starch concentration.

4.9 Retrogradation

Retrogradation of chickpea starches was determined by turbidity measurements. The extent of absorption of the gelatinized starch suspensions during storage at 40°C are presented in **Figure 4-5**. In all starches, absorption increased steeply during the first 48h of storage (CDC ICC 12512-9 ~ CDC 418-59 > CDC Xena > CDC Flip 97-133c). Thereafter, the increase in absorption was marginal. The increase in absorption during storage is a reflection of interplay of many factors such as the extent of amylose leaching, presence of unfragmented granules, rate of aggregation of leached amylose, and binding of granule remnants into assemblies by leached amylose and amylose aggregates. Consequently, it is difficult to explain the differences in absorption increase among the starches. No comparison is possible with the data available for other chickpea cultivars (Singh, Sandhu and Kaur, 2004) due to differences in starch concentration and storage temperature.

4.10 Acid hydrolysis

Hydrolysis of the chickpea starches by 2.2M HCl is presented in **Table 4-6**. All four starches exhibited a two stage solubilization pattern. A relatively higher rate was observed during the first 10 days (corresponding mainly to the degradation of the amorphous regions of the granule), followed by a slower rate (corresponding to the degradation of the crystalline region) between 10 and 20 days.

Figure 4-5: Effect of storage time on turbidity development (measured by absorbance) of four native chickpea starches

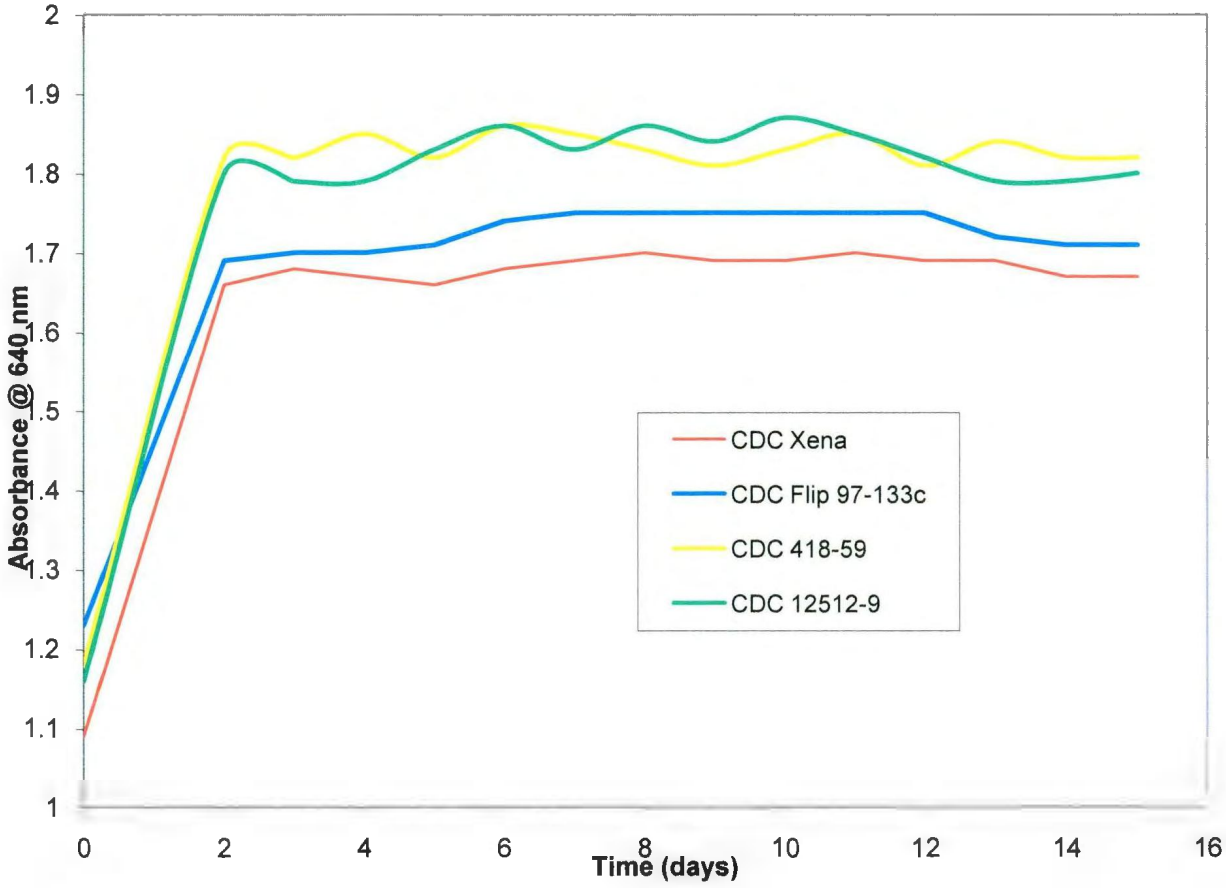

2
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
Absorbance @ 640 nm
0
2
4
6
8
10
12
14
16
Time (days)
CDC Xena
CDC Flip 97-133c
CDC 418-59
CDC 12512-9

Table 4-6: Acid hydrolysis of chickpea starches[1]

Chickpea Cultivar	Hydrolysis time (days)									
	2	4	6	8	10	12	14	16	18	20
CDC Xena	4.98±0.06^{b}	14.05±0.05^{b}	28.38±0.11^{a}	36.85±0.25^{a}	44.54±0.48^{a}	50.68±0.14^{a}	56.35±0.47^{a}	61.70±0.38^{a}	65.07±0.48^{a}	71.18±0.49^{a}
CDC Flip 97-133c	5.67±0.09^{a}	14.83±0.25^{a}	25.92±0.33^{c}	35.65±0.24^{b}	44.16±0.38^{a}	49.76±0.14^{b}	53.73±0.29^{b}	59.09±0.29^{b}	63.02±0.50^{b}	67.89±0.50^{c}
CDC 418-59	4.60±0.14^{c}	13.54±0.20^{c}	26.93±0.25^{b}	34.61±0.14^{c}	41.76±0.43^{b}	48.79±0.11^{b}	53.17±0.20^{b}	59.06±0.41^{b}	63.56±0.30^{b}	71.31±0.46^{a}
CDC ICC 12512-9	4.41±0.05^{c}	13.61±0.09^{b,c}	24.85±0.09^{d}	33.42±0.20^{d}	40.47±0.14^{c}	46.20±0.49^{c}	52.31±0.24^{c}	57.20±0.43^{c}	63.28±0.39^{b}	69.18±0.49^{b}

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

This two-stage solubilization pattern has also been reported in other pulse starches (Hoover, Swamidas and Vasanthan, 1993; Jayakody et al., 2007). The extent to which the starches degraded during the time course of hydrolysis did not follow the same trend. For instance, on day 2, days 4 to 16, day 18 and day 20, hydrolysis followed the order: CDC Flip 97-133c > CDC Xena > CDC 418-59 > CDC ICC 12512-9, CDC Xena > CDC Flip 97-133c > CDC ICC 12512-9, CDC Xena > CDC 418-59 > CDC ICC 125129 > CDC Flip 97-133c, and CDC 418-59 > CDC Xena > CDC ICC 125129 > CDC Flip 97-133c, respectively (**Table 4-6**).

Acid hydrolysis has been shown to be influenced by amylopectin chain length distribution (Chung et al., 2008a; Zhou, Hoover and Liu, 2004), lipid complexed amylose chains (Chavan et al., 1999), and amylose chain associations within the amorphous domains of the granule (Chavan et al., 1999). As shown earlier, the extent of association between amylose and/or amylose/amylopectin chains (**Table 4-5**), amount of lipid complexed amylose chains (**Table 4-1**) and granule crystallinity followed the order: CDC 12512-9 > CDC 418-59 > CDC Flip 97-133c > CDC Xena, CDC Flip 97-133c > CDC ICC 12512-9 > CDC 418-59 > CDC Xena, and CDC ICC 125129 > CDC Xena > CDC 418-59 > Flip 97-133c, respectively. Several studies (Chavan et al.; 1999; Hoover and Manuel, 1996; Kainuma and French, 1971; Morrison et al., 1993) have shown that strong associations involving amylose chains, high content of lipid complexed amylose chains and high levels of crystallinity hinder the transformation of D-glucopyranose from the chair to the half-chair conformation. This transformation has been shown (Kainuma and French, 1971) to be a pre-requisite for hydrolysis of glucosidic bonds by H_3O^+. This

suggest that differences in the extent of acid hydrolysis among the chickpea starches is influenced by the interplay of the above factors. The percentage hydrolysis of the chickpea starches at the end of 20 days (67.9 – 71.3%) was within the range reported (Chavan et al., 1999; Ratnayake et al., 2001) for other pulse starches (26.5 – 49%). No data is available for other cultivars of chickpea.

4.11 *In vitro* starch digestibility

Rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) contents of the chickpea starches are presented in **Table 4-7**. The results show that RDS, SDS and RS levels followed the order: CDC Flip 97-133c > CDC Xena ~ CDC 418-59 > CDC ICC 12512-9, CDC Flip 97-133c > CDC Xena ~ CDC 418-59 > CDC ICC 12519-9 and CDC ICC 12512-9 > CDC 418-59 ~ CDC Xena > CDC Flip 97-133c, respectively. Starches having high RDS levels tend to exhibit low RS levels and vice versa, as is demonstrated for CDC Flip 97-133c and CDC ICC 12512-9, respectively. Furthermore, SDS and RS accounts for approximately half and over a third of the total digestible starch for cultivars CDC Xena and CDC 418-59. These two types of starch (SDS and RS), important for diabetics and beneficial as potential prebiotics, account for about 89% of the total chickpea starch (excluding CDC Flip 97-133c at 84%).

Starch digestibility has been shown to be influenced by starch source, granule size, amylose/amylopectin ratio, crystallinity, amylopectin molecular structure, surface pores and interior channels (Hoover and Sosulski, 1991; Jane, Wong and McPherson,

Table 4-7:Amount of rapidly digestible, slowly digestible, and resistant starch (RS) content of native chickpea starches[1]

Chickpea Cultivar	RDS[2]	SDS[3]	RS[4]
CDC Xena	11.67 ± 0.20^{b}	50.97 ± 0.60^{b}	37.54 ± 0.99^{b}
CDC Flip 97-133c	15.68 ± 0.09^{a}	60.18 ± 0.20^{a}	24.14 ± 1.00^{c}
CDC 418-59	$11.34 \pm 0.51^{b, c}$	50.83 ± 0.70^{b}	37.83 ± 1.00^{b}
CDC ICC 12512-9	10.95 ± 0.15^{c}	48.49 ± 0.41^{c}	40.57 ± 1.12^{a}

[1]All data represent the mean of 4 replicates. Values followed by the same superscript in each column are not significantly ($P < 0.05$) by Tukey's HSD test.

[2]Rapidly digestible starch (digested within 20 min).

[3]Slowly digestible starch (digested within 20-120 min).

[4]Resistant starch (not digested even after 120 min).

1997; Sandhu and Lim, 2008; Zhang, Ao and Hamaker, 2006,). The higher levels of RDS (15.7%) and SDS (60.2%) in CDC Flip 97-133c reflects its lower crystallinity (**Figure 4-3**), larger proportion of small granules (< 10μm) (**Figure 4-1**), and higher amylose content (**Table 4-1**).

As shown earlier, AML was generally lower in CDC ICC 12512-9 than in the starches (**Table 4-3**). This is indicative of stronger interactions between amylose chains and/or amylose/amylopectin chains within the granule interior of CDC ICC 12512-9 starch. Furthermore, of the four starches, crystallinity was more pronounced in CDC ICC 12512-9 starch (**Figure 4-3**). This would then explain the higher resistant starch (40.6%) content (**Table 4-7**) in CDC ICC 12512-9 starch.

The RDS levels (11.3 to 15.7%) in the chickpea starches were generally lower than those reported for pea (18.2 to 23.8%), lentil (16.0 to 16.9%) and cultivars of other chickpea (21.5 to 29.9%) starches (Chung et al. 2008a,b). The SDS (48.5 to 60.2%) levels (**Table 4-7**) were comparable to those of pea (53.7 to 59.0%) lentil (58.3 to 62.2%) and other chickpea cultivars (45.7 to 57.7%), whereas the RS (24.1 to 40.6%) levels (**Table 4-7**) were much higher than those reported (Chung et al., 2008a,b) for pea (8.1 to 12.6%), lentil (13.0 to 13.2%) and other chickpea cultivars (8.4 to 18.4%) The results suggest that chickpea starches could prevent blood glucose levels from rising too rapidly after a meal, resulting in reduced glycemic and insulinemic responses. The RDS, SDS and RS levels of the chickpea starches cannot be compared with those reported for other pulse starches, due to differences in methodology (AACC (2000) versus Englyst et al.

(2000)) and to different time periods of hydrolysis that have been defined for measurement of RDS, SDS and RS levels.

Chapter 5: Summary and conclusions

This study showed that differences in crystallinity and physicochemical properties occur among cultivars of the same species, even under identical environmental conditions. For instance, there were minor differences in crystallinity and properties among CDC Flip 97-133c and CDC 418-59. However, CDC Xena differed significantly with respect to the other starches in terms of bound lipid content (lower), total amylose (lower), amount of lipid-complexed amylose chains (lower), proportion of small (< 10μm) granules (higher), amylose leaching (higher), enthalpy and conclusion temperature of gelatinization (higher), peak viscosity (higher), breakdown viscosity (higher) and set-back viscosity (higher), whereas, CDC ICC 125129-9 differed significantly from the other starches with respect to crystallinity (higher), swelling factor (higher), peak viscosity (lower), breakdown viscosity (lower), set-back viscosity (lower), slowly digestible starch (lower) and resistant starch (higher). The results suggest that of the four starches, CDC ICC 12512-9 is most suitable for incorporation (after minor physical or chemical modification) into foods subjected to high temperature processing, high shear, and frozen storage due to its higher crystallinity, higher thermal stability and lower set-back. Whereas, CDC Xena would require extensive physical and/or chemical modification before it could be utilized in the food industry.

Directions for Future Research

The study showed that chickpea starches in the native state are not suitable for incorporation into foods subjected to thermal processing, high shear, and repeated freezing and thawing cycles. Consequently, these cultivars may have to be physically and/or chemically modified.

Publications

Hughes, T., Hoover, R., Liu, Q., Donner, E., Chibbar, R., & Jaiswal, S. (2009). Composition, morphology, molecular structure, and physicochemical properties of starches from newly released chickpea (*Cicer arietinum* L.) cultivars grown in Canada. *Food Research International, 42*, 627-635. (citations ≠ 14)

Hoover, R., Hughes, T., Chung, H., J., & Liu, Q. (2010). Composition, molecular structure, properties, and modification of pulse starches: A review. *Food Research International, 43*, 399-413. (citations ≠ 36)

Conference Presentation

Hughes, T., & Hoover, R. (2008). Structure and properties of chickpea starches. Chemical and Biophysics Symposium, University of Toronto, April 2008.

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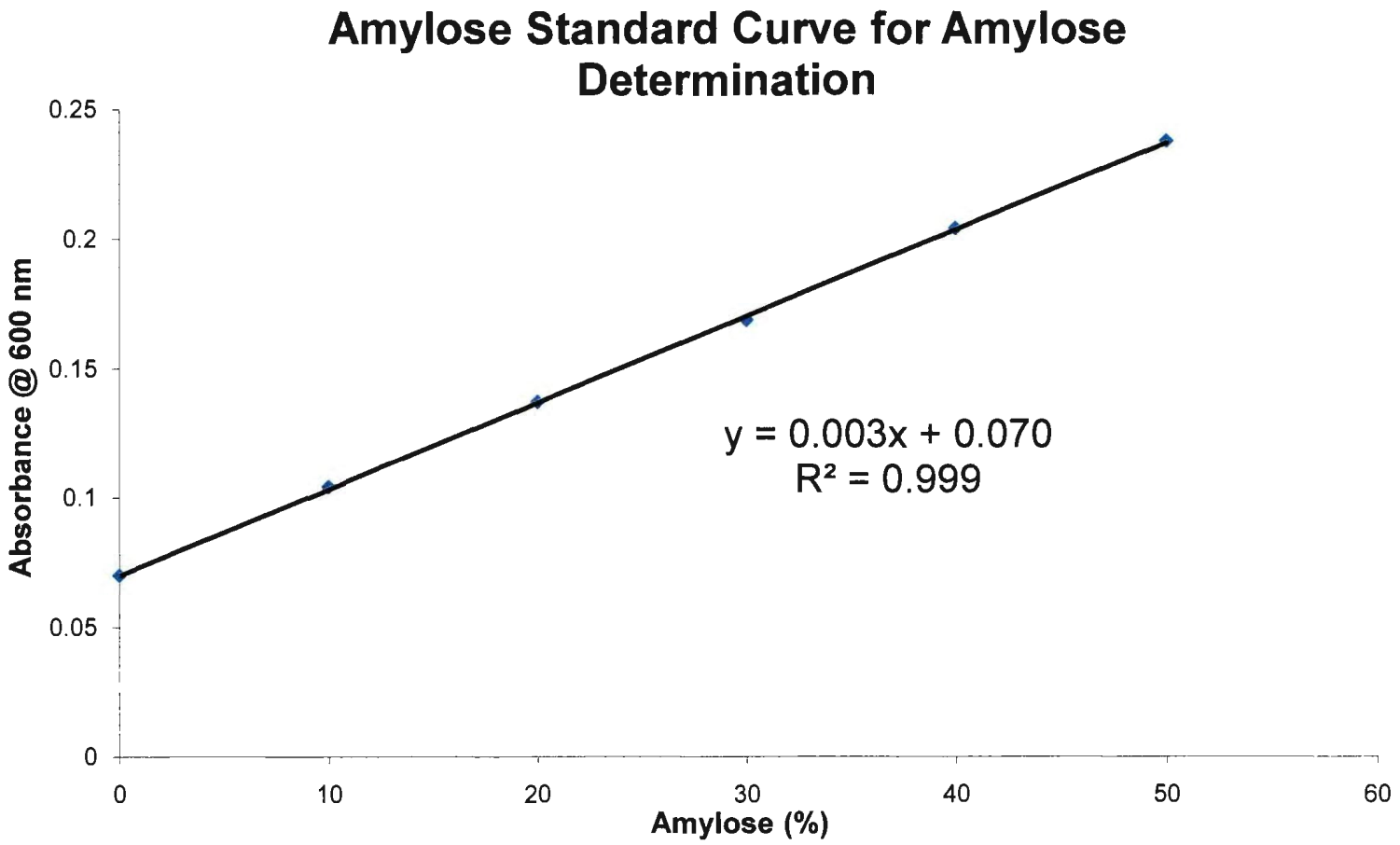
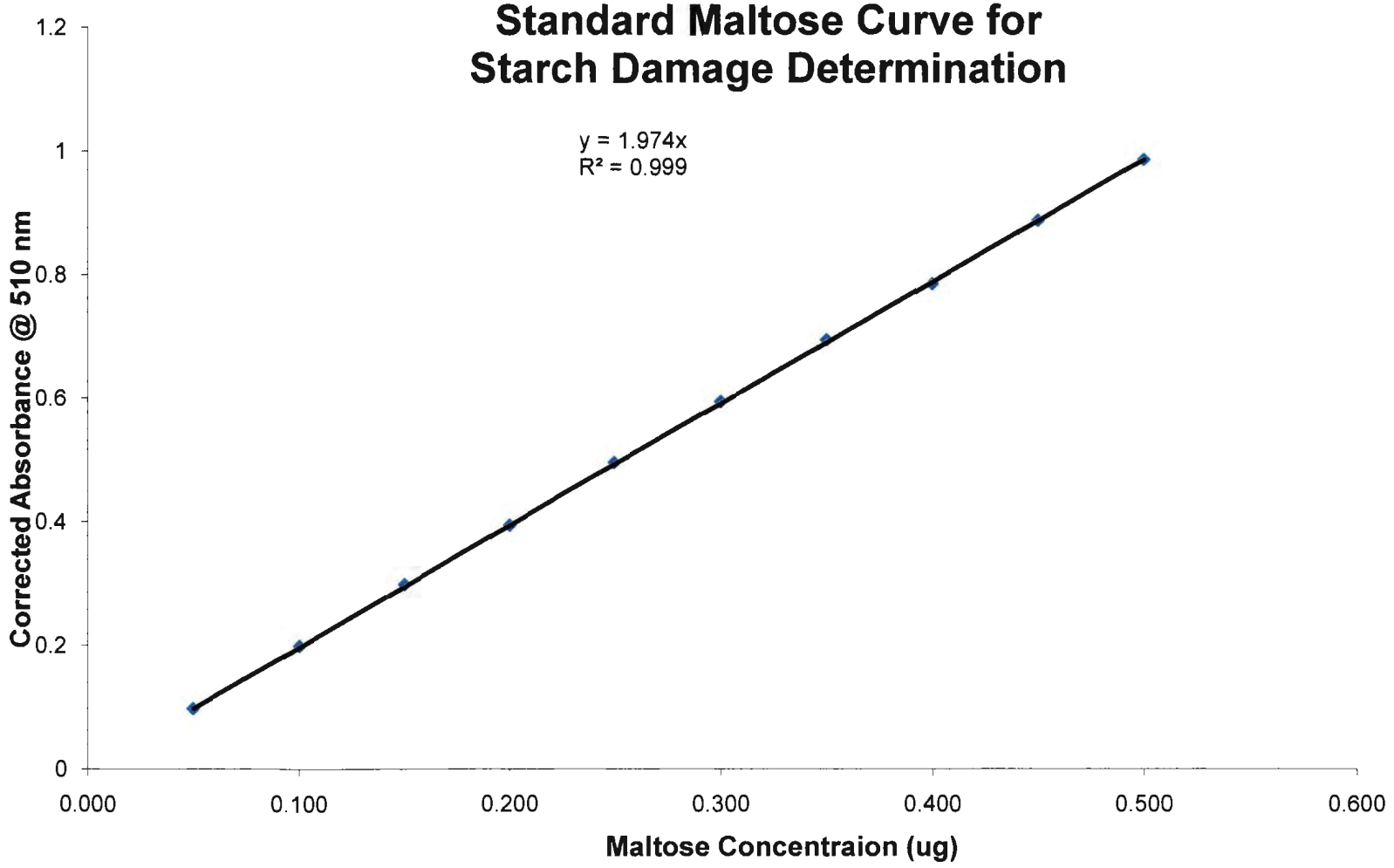
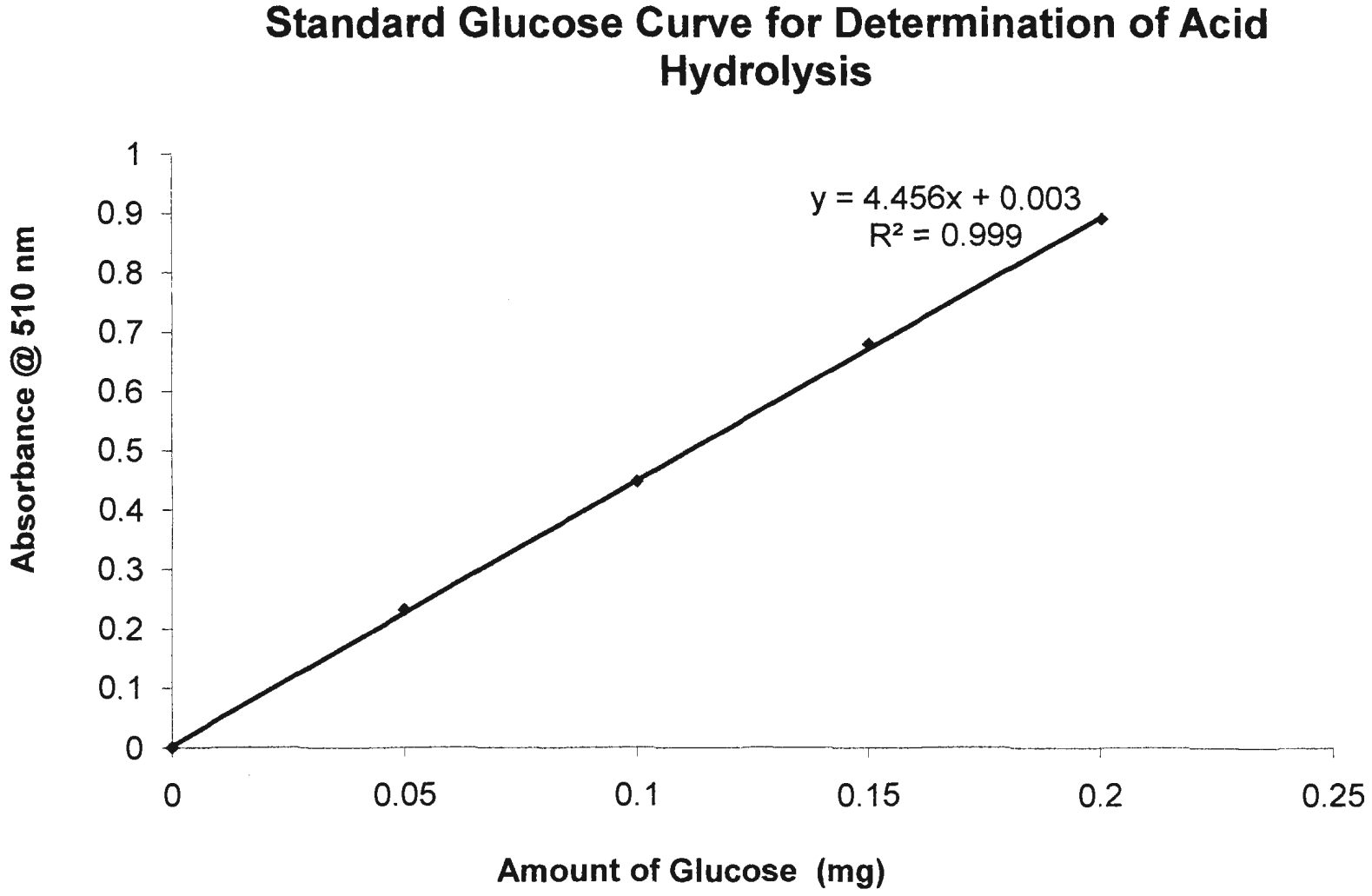
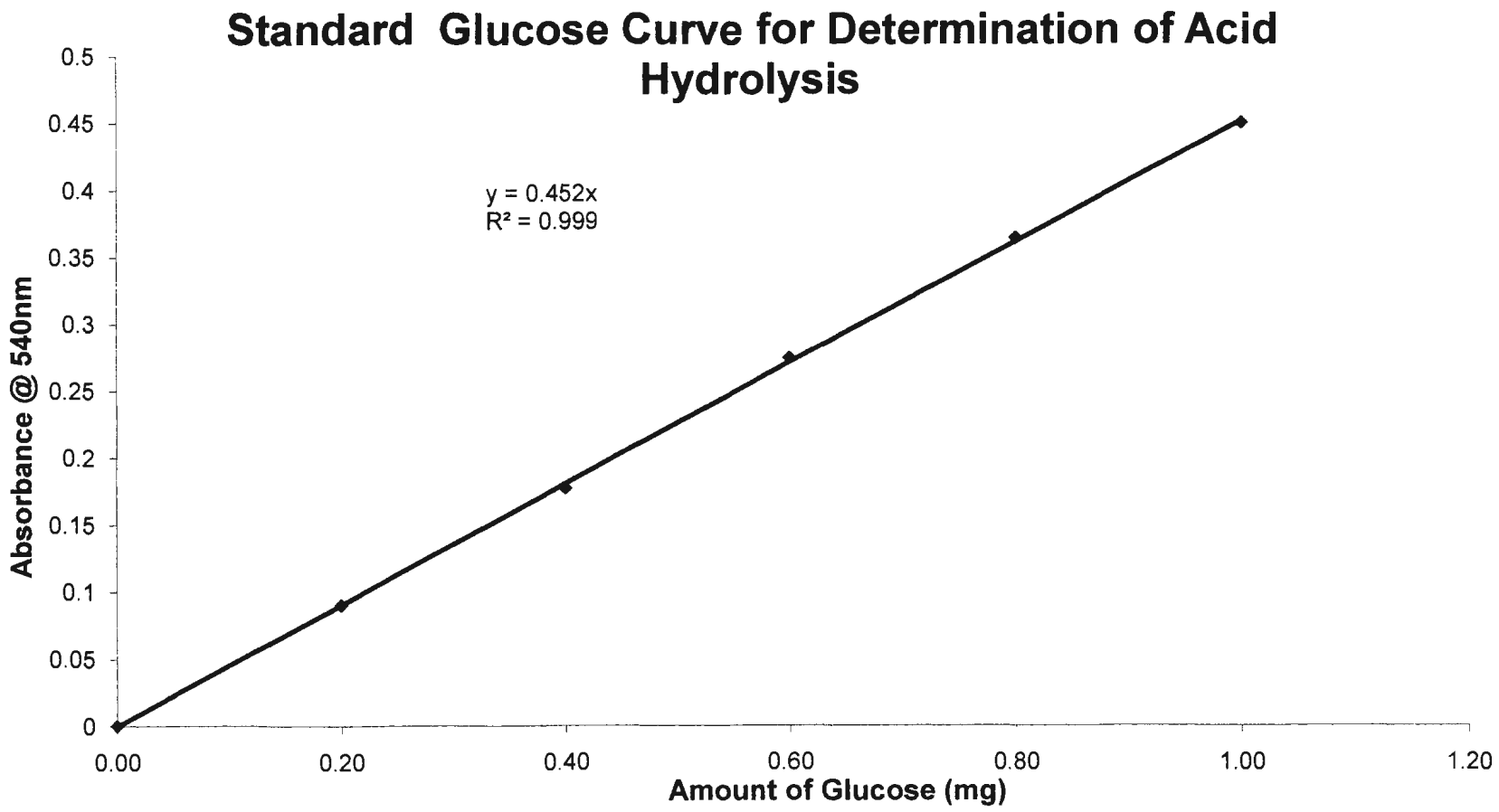
Appendix


Amylose Standard Curve for Amylose Determination
Absorbance @ 600 nm
0.25
0.2
0.15
0.1
0.05
0
0
10
20
30
40
50
60
Amylose (%)
y = 0.003x + 0.070
R² = 0.999


Standard Maltose Curve for
Starch Damage Determination
y = 1.974x
R² = 0.999
Corrected Absorbance @ 510 nm
1.2
1
0.8
0.6
0.4
0.2
0
0.000
0.100
0.200
0.300
0.400
0.500
0.600
Maltose Concentraion (ug)


Standard Glucose Curve for Determination of Acid Hydrolysis
y = 4.456x + 0.003
R² = 0.999
Absorbance @ 510 nm
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.05
0.1
0.15
0.2
0.25
Amount of Glucose (mg)


Standard Glucose Curve for Determination of Acid Hydrolysis
y = 0.452x
R² = 0.999
Absorbance @ 540nm
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0.00
0.20
0.40
0.60
0.80
1.00
1.20
Amount of Glucose (mg)