COMPOSITION, STRUCTURE, AND PHYSICOCHEMICAL PROPERTIES OF FIELD PEA (Pisum sativum L.) STARCH

R.M. WAJIRA SRINANDA RATNAYAKE

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by

© R.M. Wajira Srinanda Ratnayake
B.Sc. (Agric.) (Peradeniya)

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Abstract

Starch from four cultivars (Carneval, Carrera, Grande and Keoma) of field pea (*Pisum sativum* L.) was isolated and its composition, structure, and physicochemical properties were investigated. The results obtained were compared with those of other legume starches reported in the literature. The yield of starch was in the range 32.7-33.5% on a whole seed basis. The starch granules were round to elliptical with smooth surfaces. The free lipid was 0.05% in all starches. However, bound and total lipids ranged from 0.24-0.29% and from 0.28-0.34%, respectively. The total amylose content ranged from 48.8-49.6%, of which 10.9-12.3% was complexed by native lipid. The degree of polymerization (DP) of isolated field pea amyloses ranged from 1300-1350. The chain length distributions of debranched amylopectins of the four starches were analyzed using high performance anion exchange chromatography equipped with a post-column amyloglucosidase reactor and a pulsed amperometric detector (HPAEC-ENZ-PAD). The proportion of short branch chains of chain length DP 6-12 ranged from 16.2-18.6%. Keoma displayed a larger portion (19.4%) of long branch chains (DP>37) than the other three starches (16.2-16.9%). The average amylopectin branch chain lengths ranged from 22.9-24.2. The maximum detectable DP was higher in Keoma (71) than in the other three starches (64-65). The X-ray pattern was of 'C' type. The relative crystallinity was in the range 20.8-25.1%. The proportion of 'B' polymorphic form was higher in Keoma (25.6%) than in the other three starches (22.1 to 24.1%). There were no significant differences in swelling factor. The extent of amylose leaching at 95°C ranged from 25.20-26.85. All four starches exhibited nearly identical gelatinization transition temperatures. However, the gelatinization temperature range (T_c–T_o) followed the order: Grande ~ Keoma > Carneval ~ Carrera. The four starches showed identical pasting temperatures and exhibited only marginal differences with respect to 95°C viscosity and to the increase in consistency during the holding period at 95°C. However, the set-back viscosity for Carneval was lower than that of the other starches. There were no significant differences

in the extent of acid hydrolysis. However, susceptibility towards α-amylase followed the order: Carneva l~ Carrera ~ Grande > Keoma. The magnitude of changes in transition temperatures and enthalpy, and the broadening of the endotherm during retrogradation (24h) at different storage temperatures (4 and 40°C), followed the order: 4°C/24h > 4°C/24h + 40°C/24h > 40°C/24h. The extent of retrogradation (assessed by changes in enthalpy) followed the order: Carneval ~ Grande ~ Carrera > Keoma. There were no significant differences in the extent of freeze-thaw stability. The results showed that Keoma differed from the other three starches with respect to amylopectin branch chain length distribution, 'B' polymorphic composition, enzymatic digestibility, and degree of amylopectin retrogradation.

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List of abbreviations

ΔH	Gelatinization enthalpy
$\Delta H/AP$	Gelatinization enthalpy divided by amount of amylopectin
ΔH_R	Enthalpy of retrogradation
AML	Amylose leaching
ANOVA	Analysis of variance
BU	Brabender units
CL	Chain length
DMSO	Dimethylsulfoxide
DNS	3,5 dinitrosalycylic acid
DP	Degree of polymerization
DSC	Differential scanning calorimetry
EC	Enzyme code
GPC	Gel permeation chromatography
HPAEC-ENZ-PAD	High performance anion exchange chromatography equipped with an on-line enzyme (amyloglucosidase) reactor and a pulsed amperometric detector
M	Molarity (Molar)
NMR	Nuclear Magnetic Resonance
SD	Standard deviation
SEC	Size exclusion chromatography
SEM	Scanning Electron Microscopy
SF	Swelling factor
T_c	Conclusion temperature
T_o	Onset temperature
T_p	Peak (mid-point) temperature
v/v	Volume/volume
w/v	Weight/volume
w/w	Weight/weight

1 INTRODUCTION

Legumes are dicotyledonous seeds of plants that belong to the family *Leguminosae* [16000 – 19000 species in ~750 genera] (Allen and Allen, 1981). The grain legumes collectively are grown throughout the world and ranked fifth in terms of annual world grain production (171 million metric tons) (FAO, 1999). They are mainly produced because of their high protein value (in the seeds). The major cultivated legumes include various species of peas and beans. Pea comprises two species, *viz*; *Pisum sativum* L. [cultivated peas] and *Pisum fulvum* L. [wild peas]. Field pea (*Pisum sativum* L. ssp. *arvense*) -which is also known as common pea, dry pea, green pea [green seeded cultivars], yellow pea [yellow seeded cultivars], and garden pea- is a cool season crop grown in the sub-tropics and at higher altitudes in the tropics (Allen and Allen, 1981). It is also one of the four important cultivated legumes (the others being soy bean, groundnut and dry bean) worldwide. Pea is a predominant export crop in world trade and represents about 35-40% of the total trade in pulses. In 1999, Canada contributed 19% to the total world production of 11,699,171Mt (FAO, 1999), and Canadian pea production increased by 30% in the 1998/99 season (Agriculture and Agri-Food Canada, 2000). Canada's largest export market for field pea is Western Europe. Field pea is utilized in Canada as follows: (1) whole or split in soups and stews; (2) hulls in high fiber breads; (3) pea protein in human food and in hog rations as an alternative protein source to soy and canola meal; and (4) pea starch in production of adhesives and carbonless paper (Agriculture and Agri-Food Canada, 2000). The air-classification and wet-milling process for field

pea (Tyler *et al.*, 1981) has permitted the fractionation of field pea flour into protein and starch concentrates. However, due to its relatively low starch content and the lack of information on its functional properties, the starch concentrate is currently not being utilized in food formulations. Therefore, it is economically important to explore possible avenues of improving the functional properties of field pea starch for it to be successfully utilized in the food industry.

Total carbohydrates of food legumes vary from 24% (winged beans) to 68% (cowpeas). Starch is the most abundant legume carbohydrate and the amount varies from 24% (wrinkled peas) to 56.5% (pinto beans) (Reddy *et al.*, 1989). Starches from different cultivars of wheat (Wootton and Mahder, 1993), maize (Yun and Matheson, 1993), proso millet (Yanez *et al.*, 1991), rye (Gudmudsson and Eliasson, 1991) and cassava (Asaoka *et al.*, 1991) have been shown to vary in composition and properties. However, such variations in starch composition and properties among field pea cultivars have not been the subject of a detailed study. Most of the studies on field pea starch have been on a single cultivar. Therefore, it is difficult to ascertain whether the data reported is truly representative of field pea starch. Furthermore, there is a dearth of information on the retrogradation properties of field pea starch. Therefore, it was considered worthwhile to investigate the chemical composition, granule crystallinity, polymorphic composition, thermal properties, rheological properties and retrogradation characteristics of starches isolated from four cultivars of field pea which were grown under identical environmental conditions. At the present time,

there is increased emphasis on developing value-added products from Canadian legumes, particularly from pea starch, which is the major by-product of pea protein isolation. The results of this study would form the basis for further investigations on physical and chemical modification to improve the functionality of field pea starches.

2 Literature review

2.1 Starch – General information

Starch is the major form in which carbohydrates are stored in higher plants. Starch in the chloroplasts is considered to be transitory; it accumulates during the light period and is utilized during the dark (Shannon and Garwood, 1984). Starch occurs as water insoluble granules and the size, distribution, morphology and shape of those granules vary with the species and maturity of the plant (Manners, 1989; Snyder, 1984). In higher plants, starch occurs in leaves, stems (pith), woody tissues, roots, tubers, bulbs, rhizomes, seeds (pericarp, cotyledon, embryo, and/or endosperm), fruits, flowers and/or pollen. Food starches are derived mainly from seeds (*e g.* wheat, maize, rice, barley, etc), root (cassava) or tuber (potato). Starch serves as the main source of carbohydrate food for many species of animals. It is also the main carbohydrate in almost all the major foods used by man. Most of the starches utilized worldwide come from a relatively smaller number of crops, the most important being maize, potato, wheat and tapioca. Relatively smaller amounts come from rice, sorghum, sweet potato, arrowroot, sago and mung bean (Wang *et al.*, 1998).

Chemically, starch is a large polymer of glucose consisting of glucose units formed into unbranched amylose chains composed of α(1-4) linked glucose residues and highly branched amylopectin with α(1-4) and α(1-6) bonds (Scott *et al.*, 1999). Although, amylose is defined as a linear molecule, it is now known that some amylose molecules have several branches. Furthermore, the

presence of an intermediate material (between amylose and amylopectin) has been reported in amylomaize and wrinkled pea starches (Hizukuri, 1996).

2.2 Starch production and utilization

Starch occurs throughout the plant world. However, for the commercial production of starch, only a limited number of plants are used. For instance, about 97% of the starch produced in United States comes from maize. Relatively smaller amounts are produced from potato and wheat. However, in Europe, the production of potato starch is much higher compared to that in the United States, and wheat starch is also produced abundantly. Most of the European starch production (>70%) is from maize. Tapioca and sago starches are produced in tropical countries such as Brazil and East Indies. Tapioca starch is mainly used for industrial and food applications and for the production of modified starches. Arrowroot starch is produced in African and Caribbean regions (Wurzburg, 1986).

Different processes are used in the manufacture of starch depending on the plant source. In general, all of these processes involve the separation of starch granules from other constituents (*e g.* fiber, germ, protein, and other extraneous material), purification, washing, and drying (Wurzburg, 1986).

In Canada, starch related food processing uses mainly corn, wheat, field pea, and potato as sources. Corn starch is the major form of starch utilized in Canada. Wet milling is mainly used to produce corn starch, and about one

million tons per year of corn is processed by this technique. Seventy five percent of the starch produced in Canada is used for industrial purposes (~80% of which is used for paper and corrugated box manufacturing), and the balance is used in the food industry. The proportion of the total production of starch used for industrial purposes is 70% in the United States and 69% in Europe (Industry, Science and Technology Canada, 1990; Lillford and Morrison, 1997).

Nearly 75% of the Canadian starch production is centered in Ontario, New Brunswick, Quebec, Saskatchewan and Manitoba. Field pea starches are mainly produced in Manitoba and Saskatchewan whereas potato starch is produced mainly in New Brunswick. The byproducts of pea starch production such as pea fiber and protein are used in dietetic foods, fortified breads, industrial processes, and animal feed supplements (Industry, Science and Technology Canada, 1990).

The main food and industrial uses of starch and its derivatives are listed in **Table 2-1**. Small granular and microcrystalline starches are used as fat substitutes in food industry. The use of starch in various non-food products also depends on the granular shape and size. Mainly in paper and textile industries, starch pastes are used as binding and sizing agents. Large granuled wheat starch is used in the production of carbonless copy paper. Small granuled starch (such as amaranth, small wheat and rice) is preferred for the preparation of flavor carriers (Jane, 1997).

Table 2-1 - Some of the main uses of starch and its derivatives (adopted from Galliard, 1987; Lillford and Morrison, 1997).

Food uses	Industrial uses
Functions: Thickening, gelling, stabilizing, sweetening, bulking, texturizing, fat replacement Products: Sauces, soups, dressings, baked foods, dairy products, pie fillings, gums, jellies, meat products, drinks, ice cream, instant-product coatings, frozen-food, infant formulae.	Functions: Sizing, coating, texturizing, viscosity control, flocculation, ion exchange, adhesive, dusting, fuel, metal refining, metal sequestrants. Products: Paper and board, textiles, plastics, rubber, oil, pharmaceuticals, medicine, cosmetics, adhesives.

The physical properties (such as lack of free-flowing properties, insolubility or failure of the granules to swell and develop viscosity in cold water, excess or uncontrolled viscosity after cooking, cohesive or rubbery texture of the cooked starch, the sensitivity of the cooked starches to breakdown during extended cooking, when exposed to shear or to low pH, *etc.*) of native unmodified starches make them unsuitable for many food applications. Modified starches have been developed to overcome these shortcomings and to expand the usefulness of starch (Wurzburg, 1986). Different methods are used to improve the properties of starch paste by chemical, physical, enzymatic and genetic modifications (Jane, 1997). Common methods commercially used for starch modification include derivatizing, cross-linking, hydrolyzing and oxidation (Wurzburg, 1986).

2.2.1 Importance of legumes as a source of starch

The family *Leguminosae* contains about 16,000-19,000 species in about 750 genera with worldwide distribution. In terms of economic importance, it is second only to family *Graminae* (grasses) (Allen and Allen, 1981). The total world pulse production was 59,275,083Mt in 1999. This amount includes dry beans (32.7%), dry peas (19.7%), chick peas (15.6%), and small amounts of cow peas, pigeon pea, lentils, bambara beans, vetches, lupins, *etc.* (FAO, 1999). Starch isolation from legume seeds is difficult due to the presence of insoluble flocculent protein and fine fiber, which co-precipitate with starch (Schoch and Maywald, 1968; Hoover and Sosulski, 1985). Legume starches can be isolated both by aqueous sedimentation and pin-milling and subsequent air-classification techniques

(Reichert and Youngs, 1978; Schoch and Maywald, 1968; Tyler *et al.*, 1981). Legume seeds contain considerable amounts of carbohydrate although they are primarily grown for their protein value. The legumes are mainly cultivated for their protein value. Most of the cultivated legumes contain more than 20% (w/w) protein while the total carbohydrate content varies from 25 to 64% in the seeds (Agustin and Klein, 1989). The proximate compositions of some cultivated legumes and their isolated starches are given in **Table 2-2** and **Table 2-3**, respectively.

2.2.2 Peas

There are two types of cultivated peas, *i e.*, garden peas (*Pisum sativum* ssp. *hortens*) and field peas (*Pisum sativum* ssp. *arvense*) (Adsule *et al.*, 1989). Field pea is a crop plant whose utilization can be traced back to neolithic times (Zohary and Hopf, 1988). It is a cool season food legume/pulse and widely grown in the temperate zones of the world. Field pea seeds are a rich source of protein, carbohydrate and minerals (Adsule *et al.*, 1989). The composition of dry pea seeds is given in **Table 2-4**. The carbohydrate portion includes sugars (mono-, di- and oligo-saccharides) and starch. However, the amount of sugars in the seeds is very low (Adsule *et al.*, 1989). The amounts of different carbohydrates in pea are given in **Table 2-5**.

Table 2-2 - Proximate composition (%) of some cultivated legumes (Deshpande and Damodaran, 1990).

Legume	Carbohydrate	Protein	Fat	Moisture	Fiber	Ash
Chick pea	11.7	30.4	47.7	5.4	2.5	2.3
Garden pea	28.0	22.5	1.0	10.6	3.4-12.5	3.9
Cow pea	56.8	23.4	1.3	11.0	3.9	3.6
Soy bean	14.0-23.9	29.6-50.3	13.5-24.2	5.0-9.4	2.8-6.3	3.3-6.4
Lentil	55.8	25.0	1.0	11.2	3.7	3.3
Pigeon pea	57.3	19.2	1.5	10.1	8.1	3.8

Table 2-3 - Composition (%) of some isolated legume starches (Hoover and Sosulski, 1991).

Source	Starch	Protein	Lipid	Ash
Chick pea	40	0.70-0.94	0.06	0.07
Smooth pea	40	0.52-0.70	0.01-0.10	0.07
Cow pea	37	0.12-0.50	0.21-0.33	0.06
Lentil	25-42	0.17-0.53	0.05-0.23	0.13
Kidney bean	25	0.13-0.30	0.18	0.18

Table 2-4 - Proximate composition of dry pea seeds (Adsule *et al.*, 1989).

Constituent	Content (%)
Moisture	16.0
Carbohydrate	56.5
Protein	19.7
Fat	1.1
Minerals	2.2
Crude fiber	4.5

Table 2-5 - Carbohydrate content of peas (Reddy *et al.*, 1984).

Constituent	Content (%)
Total sugars	5.3 – 8.7
Sucrose	2.3 - 2.4
Raffinose	0.3 - 0.9
Stachyose	2.2 - 2.9
Verbascose	1.7 - 3.2
Starch	36.9 - 48.6
Cellulose	0.9 - 4.9
Hemicellulose	1.0 - 5.1
Lignin	0.5 - 0.9
Total carbohydrates	56.6

Peas are produced in almost all countries in the world. They are ranked fourth in terms of the world production of food legumes below soybeans, peanuts and dry beans (FAO, 1982). Since 1993, Canadian pea production has increased significantly (Agriculture and Agri-Food Canada, 1998). Canada is the second largest producer (~19% of total world production) and the largest exporter (40% of total world exports) of dry pea in the world (Agriculture and Agri-Food Canada, 2000). However, more than half of the total Canadian pea production is exported as seed without further processing. In Canada, dry pea is not utilized at a large scale in industry. About 10% is used as seed, whole or split seeds are used in stews, soups and canned food, hulls are used in high fiber breads and some amount is used in animal feed (Agriculture and Agri-Food Canada, 1998, and 2000). Dry peas are mainly grown for protein isolation. An air classification technique is used to separate the protein and starch fractions in field pea (Tyler *et al.*, 1981; Vose *et al.*, 1976). Tyler *et al.* (1981) reported that the process of double pass pin milling and subsequent air classification separated field pea flour into starch (~60% of the yield) and protein fractions. However, this starch is not being used in food products. A small amount of pea starch is used in adhesives and carbonless paper production (Agriculture and Agri-Food Canada, 1998).

The starch in pea is present in the parenchymatous tissue of the seed cotyledons. There are two main classes of pea starch – low and high amylose. The starches from smooth seeded peas have lower amylose contents (29-35%), whereas those from wrinkled peas have higher contents (66-90%) of amylose

(Adsule *et al.*, 1989). Gujska *et al.* (1994) reported that the high amylose content of the starch made the field pea more suitable for extrusion cooking compared to other legume starches. They also reported that field pea starch granules were relatively larger in size than those of some other legume starches.

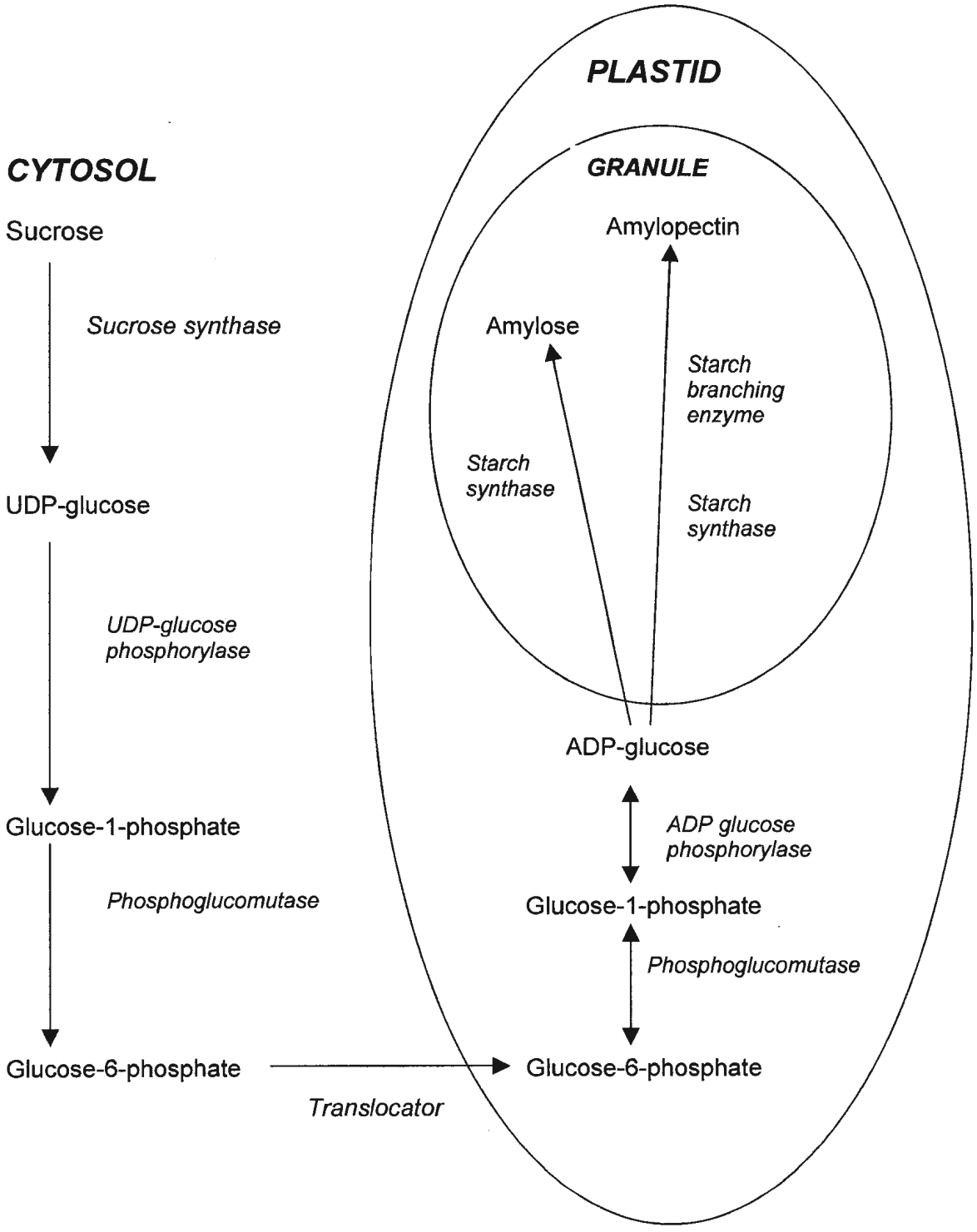
Biliaderis (1982b) reported that the *in vitro* digestibility of smooth pea starch by porcine pancreatic α-amylase was 71.4% in 5h. This is much lower than that reported for other legume starches such as: kidney bean – 31.4% in 6h, black bean – 34.8% in 6h, and pinto bean – 25.2% in 6h (Hoover and Sosulski, 1985). The differences in *in vitro* digestibility of native starches are attributed to the interplay of various factors such as granule size (Snow and O'Dea, 1981; Ring *et al.*, 1988), amylose/amylopectin ratio (Dreher *et al.*, 1984; Hoover and Sosulski, 1985; Ring *et al.*, 1988), degree of crystallinity (Dreher *et al.*, 1984; Hoover and Sosulski, 1985); amylose chain-length (Jood *et al.*, 1988), amylose/lipid complexes (Holm *et al.*, 1983), etc. Wursh (1986) reported that starch from pea was more slowly digested than starches from other sources such as beans and lentils. This was attributed to differences in granular swelling (pea > beans ~ lentils). Compared to rice starch (100), the rates of digestion of yellow pea (32) and green pea (52) starch were very slow. This might be an important nutritional characteristic, which may enable the use of pea starches in dietetic foods.

2.3 Starch biosynthesis

Although the exact mechanism by which plant genetic information is translated into specific granule composition and organization is not known, it has been reported that granule composition, morphology and supermolecular organization are, to some extent, under genetic control (Banks and Greenwood, 1975; French, 1984).

The complete pathway of starch biosynthesis in plants is complex and not completely understood (Shannon and Garwood, 1984). Erlander (1958) first reported that the biosynthesis of starch is preceded by the production of a precursor plant glycogen and this precursor is then debranched to produce amylopectin and amylose. This theory was further confirmed by subsequent findings such as the presence of debranching enzymes in corn endosperm (Lee *et al.*, 1971) and the action of debranching enzymes for the production of amylose and amylopectin from plant glycogen (Mouille *et al.*, 1996; Ball *et al.*, 1996; Erlander, 1998a, b). It is believed that in the biosynthesis of amylopectin, granule-bound starch synthases regulate α(1-4) linkage formation, and branching enzymes in a transferase reaction regulate α(1-6) branch point formation. It is widely accepted that three main enzymes are involved in the synthesis of starch in the amyloplast. They are: (1) ADP-glucose phosphorylase, (2) starch synthase, and (3) starch-branching enzyme (Wang *et al.*, 1998). A generalized pathway proposed for the production of starch is shown in **Fig. 2-1**. The exact roles of the starch biosynthetic enzymes, such as phosphorylase,

Fig. 2-1 – A proposed pathway of starch biosynthesis (modified from Wang *et al.*, 1998)


PLASTID
CYTOSOL
GRANULE
Sucrose
Amylopectin
Amylose
Sucrose synthase
Starch branching enzyme
Starch synthase
Starch synthase
UDP-glucose
UDP-glucose phosphorylase
ADP-glucose
ADP glucose phosphorylase
Glucose-1-phosphate
Phosphoglucomutase
Glucose-1-phosphate
Phosphoglucomutase
Glucose-6-phosphate
Glucose-6-phosphate
Translocator

starch synthases and branching enzymes, are yet to be fully investigated (Biliaderis, 1998).

2.4 Starch structure and composition

Starch granules mainly contain a mixture of amylose and amylopectin, the latter being the main component, which is composed of extensively branched macromolecules (Manners, 1989). The approximate amounts (w/w) of amylose and amylopectin in starch are 15-30% and 85-70%, respectively, except in genetically modified waxy (0-8% amylose) and high-amylose (50-70% amylose) starches. Both amylose and amylopectin are heterogonous molecules and exhibit a wide distribution of molecular structures and sizes (Biliaderis, 1998; Lillford and Morrison, 1997).

Many physical techniques are available to analyze starch structure. Among these, wide-angle X-ray diffractometry, small angle X-ray diffractometry, solid-state ^{13}C-NMR, various viscometric techniques and differential scanning calorimetry (DSC) are the most widely used. Each of these methods gives information about different aspects of starch structure (Biliaderis, 1998).

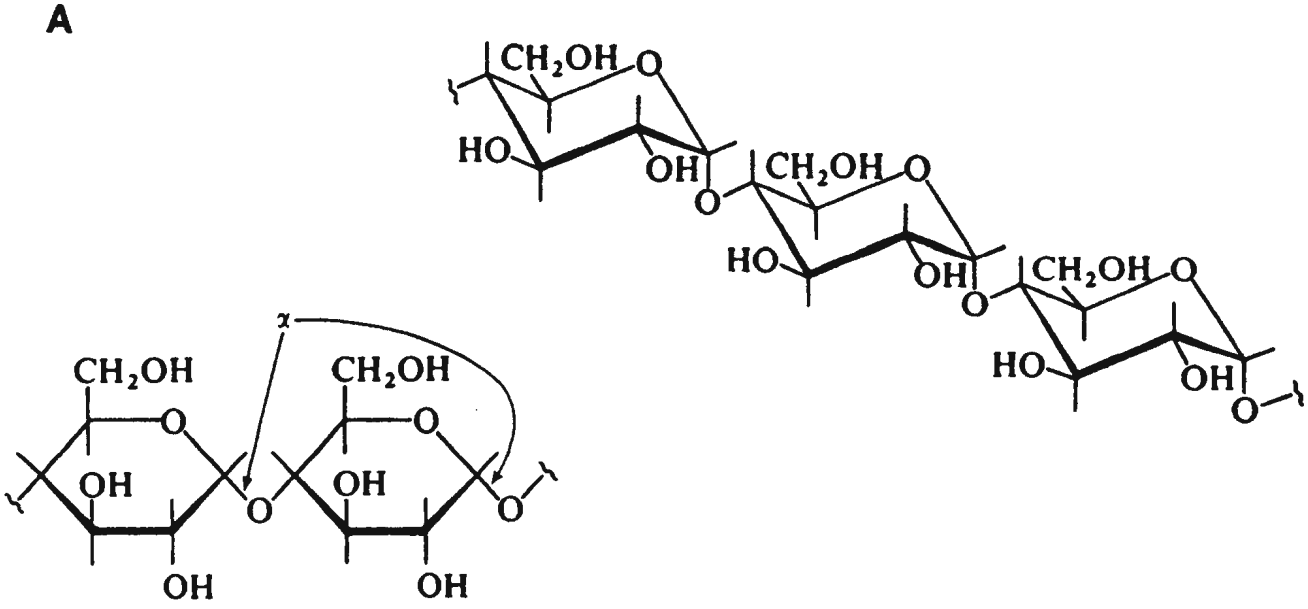
2.4.1 Major components of starch

2.4.1.1 Amylose

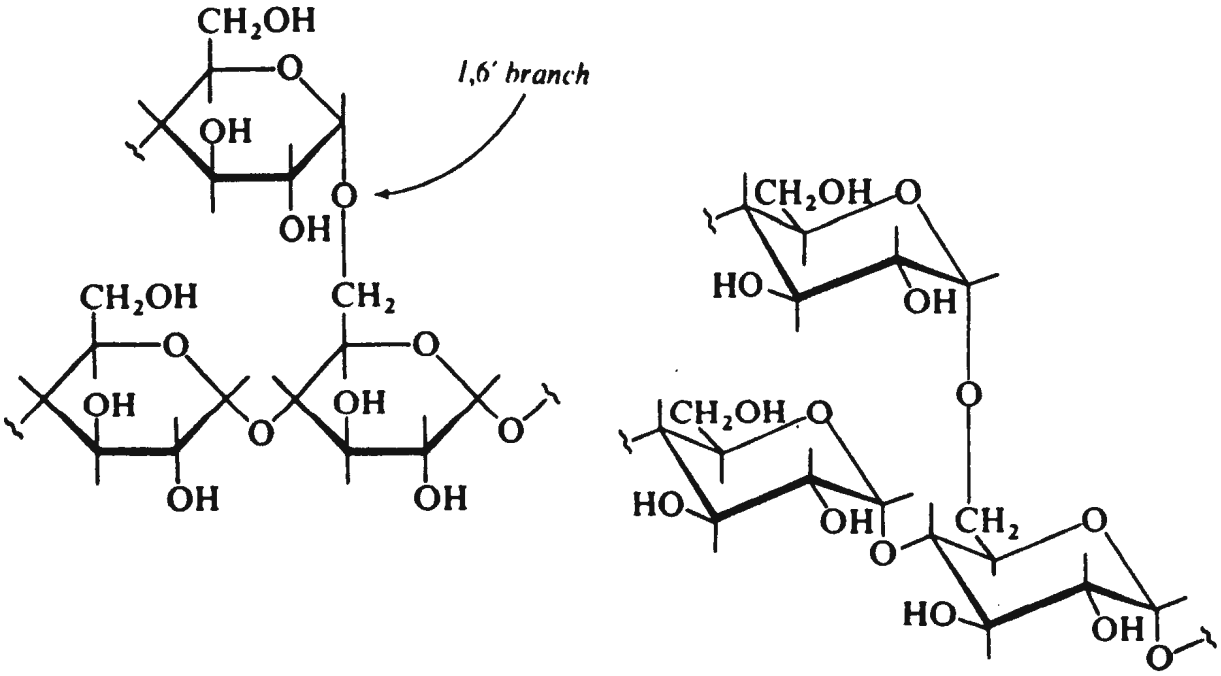
Amylose is often referred to as the linear starch fraction and contains mainly α(1-4) linked D-glucopyranosyl residues (**Fig. 2-2**). However, it was later found that it

Fig. 2-2 – The arrangement of anhydroglucose units in the molecular structures of (A) amylose and (B) amylopectin. (Fessenden and Fessenden, 1986 – with permission from Brooks/Cole Publishing Co.).

A



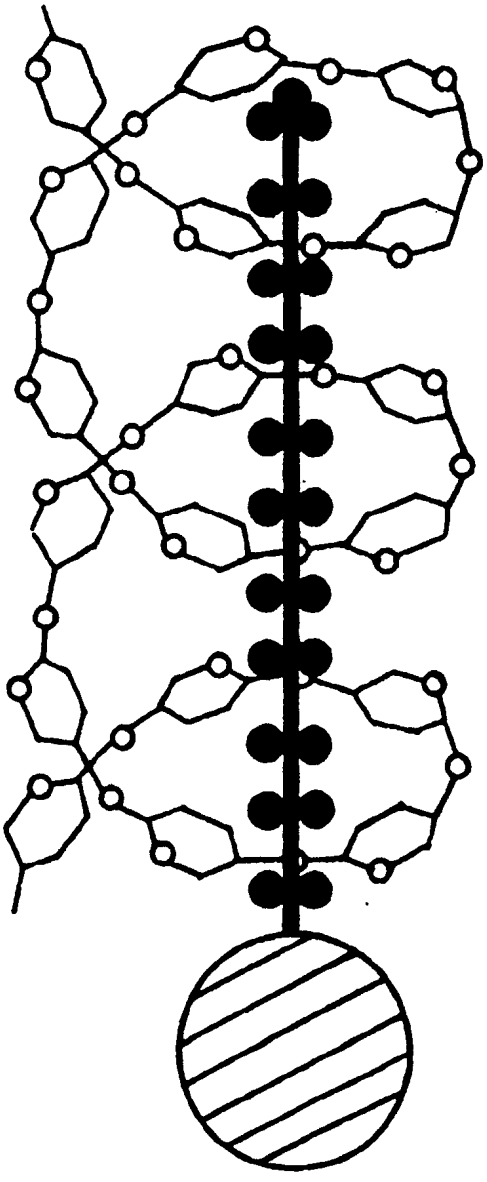
B



has occasional α(1-6) branch points (9-20 branch points per molecule) and the branch chains have chain lengths in the range from 4 to >100 glucosyl units (Hizukuri *et al.*, 1981). Amylose has an average degree of polymerization (DP) of approximately 1000-6000 (Lineback, 1999; Zobel, 1988a) whereas amylopectin has a DP range of $3x10^5$ – $3x10^6$ (Zobel, 1988a). Studies have shown that the molecular weights of laboratory-isolated amyloses range from $2.0x10^5$ to $1.2x10^6$Da. Despite its slightly branched nature, in neutral solutions and other solvents such as DMSO, formamide and aqueous alkali, amylose behaves as a random coil, assuming a helical conformation in the presence of a complexing agent (Banks and Greenwood, 1971 and 1975) (**Fig. 2-3**).

Amylose tends to be present in higher amounts in the periphery than in the center of the granules. This phenomenon has been supported by the facts that the iodine affinity, $\lambda_{max.}$ (Maximum wavelength of the spectrum of iodine coloration), and the fractions corresponding to amylose in CL distribution analysis have been reported to increase during starch granule development in some normal maize (Inouchi *et al.*, 1984), wheat (Duffus and Murdoch, 1979), barley (Banks *et al.,* 1973), rice (Asaoka *et al.*, 1985), pea (Biliaderis, 1982a) seeds and potato tubers (Geddes *et al.*, 1965). However, Jane *et al.* (1992), using cross-linking and subsequent GPC profile analysis, showed that amylose was interspersed among amylopectin molecules instead of being in bundles in the granule.

Fig. 2-3 – A schematic illustration of monostearin-amylose helical complex [the hydrocarbon chain of the monostearin stays inside the amylose helix while polar region stands outside] (Carlson *et al.*, 1979 – with permission from Wiley-VCH).



2.4.1.1.1 Complex formation by amylose

There are two main features of amylose in solution that are important to its functionality:

(a) The formation of inclusion complexes with iodine, aliphatic primary alcohols, lipids and surfactants.

(b) Its ability to form strong intermolecular interactions, *i e.*, hydrogen bonding, which leads to gel formation or precipitation when a solution of amylose is cooled (Lineback, 1999).

Starch dispersions stained with iodine give rise to the typical deep blue color (λ_{max} = 640nm) due to the formation of inclusion complexes between amylose and iodine. This facilitates the quantitative determination of amylose in I_2/KI solution. The complex molecules formed are known as polyiodide ions, *i e.*, generally I_3^- or I_5^- (Banks and Greenwood, 1975). Amylose can bind some 20% of its weight of iodine (Biliaderis, 1998). Amylose can also form complexes with a variety of other polar and non-polar compounds in aqueous solution. In each case, the amylose molecule adopts a 'V-helix' conformation where the ligand molecules lie within the helix [as shown in **Fig. 2-3**] (Banks and Greenwood, 1975). Interchain hydrogen bonds between the adjacent glucosyl residues, interturn hydrogen bonds and many other intra- and intermolecular van der Waals contacts stabilize the amylose helix (Biliaderis, 1998). Studies have shown that only the H-5 atoms of each glucopyranose residue participate in van

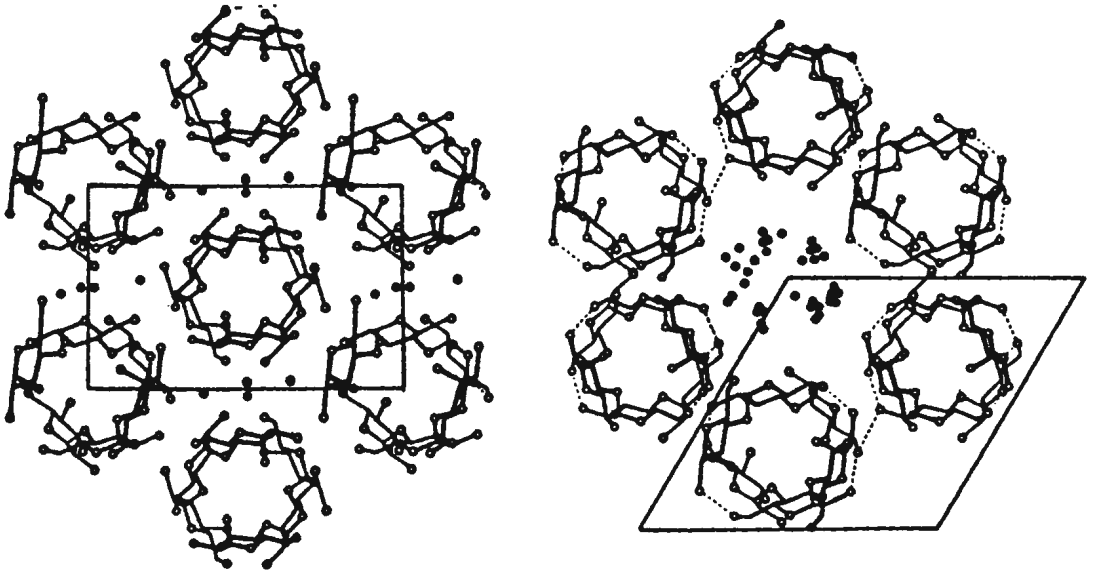
der Waals forces with the nearest CH_2 group of the aliphatic chain of the fatty acid (Godet *et al.*, 1993).

Amylose can form interchain associations mediated by local ordering of the chains to form interchain double helices. These double helices are formed over chain segments of less than 100 units. Three types (A, B and C) of helix packing have been reported for amylose. **Fig. 2-4** shows the helix packing of A and B types. The double helices of both A and B-amylose are known to be identical in conformation. Both are right handed and parallel stranded with O6 in the rotational position. However, 'A' and 'B' structures differ in crystalline packing of helices and water content. 'A' amylose crystallizes with an orthogonal unit cell with a slightly distorted hexagonal packing and with 8 water molecules per unit cell whereas 'B' amylose crystallizes in a hexagonal unit cell with an open hexagonal packing and with 36 water molecules per unit cell. The 'C' type is known to be a mixture of 'A' and 'B' amylose unit cells and therefore has an intermediate packing density (Sarko and Wu, 1978). The concentration in solution and chain length determine the solubility, molecular association and crystallization of amylose (Biliaderis, 1998).

2.4.1.2 Amylopectin

Amylopectin (**Fig. 2-2** and **Fig. 2-5**) is one of the largest naturally occurring polymers and has a molecular weight in the range 10^7-10^9 (Banks and Greenwood, 1975). The linear chains of amylopectin, containing an average of

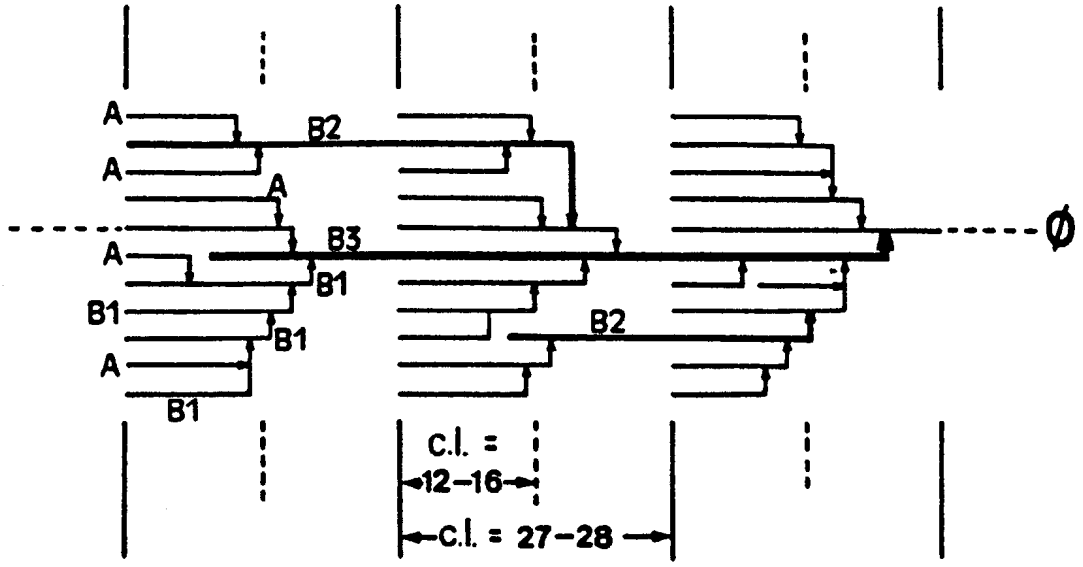
Fig. 2-4 – Unit cells and helix packing in A- [**A**] and B-amylose [**B**] (Wu and Sarko, 1978 – with permission from Elsevier Science).



B

A

Fig. 2-5 – Cluster model of amylopectin [c.l. = chain length, ∅ = reducing end of the main 'C' branch] (Hizukuri, 1986 – with permission from Elsevier Science).


A
B2
A
A
B3
A
B1
B1
B1
A
B1
B2
Ø
C.I. =
12–16
C.I. = 27–28

20-25 α(1-4) linked D-glucose residues, are interlinked by α(1-6) D-glycosidic linkages to form a highly branched structure. The presence of interchain linkages (4-5% of total) has a significant effect on the physical and biological properties of amylopectin. Various parameters, such as average chain length, exterior and interior chain lengths, ratio of A-chains to B-chains and chain profile, can be used to describe the structure of amylopectin (Manners, 1989).

Due to their branched nature, amylopectins have relatively low intrinsic viscosities (120-200mL/g) although they have high molecular weights (Banks and Greenwood, 1975). Amylopectins have a wide distribution of chain lengths with the average chain length being 20-25 units (Hizukuri, 1986) and size-exclusion chromatography (SEC) has been used to estimate CL distribution. Hizukuri (1986) found that the branch chain lengths of amylopectin show a bimodal distribution (long 'B' chains – F_1 and short 'B' and 'A' chains – F_2) by SEC. According to the model proposed by Hizukuri (1986) (**Fig. 2-5**), there are three main types of chains in the amylopectin molecule. They are: (1) 'A' chains, which are unbranched and linked to the molecule by their reducing end, (2) 'B' chains (B_1-B_4), which are connected to the molecule by the reducing end but carry one or more 'A' chains, and (3) one 'C' chain, which acts as 'the stem' and carries the reducing end of the molecule. He also found a good correlation between the weight-average CL and the ratio of weight fractions (F_2/F_1), implying that the average CL is primarily dependent on the amounts of two fractions. These F_1 and F_2 fractions were further separated into A, A_1, B_1, B_2, B_3 and B_4 fractions

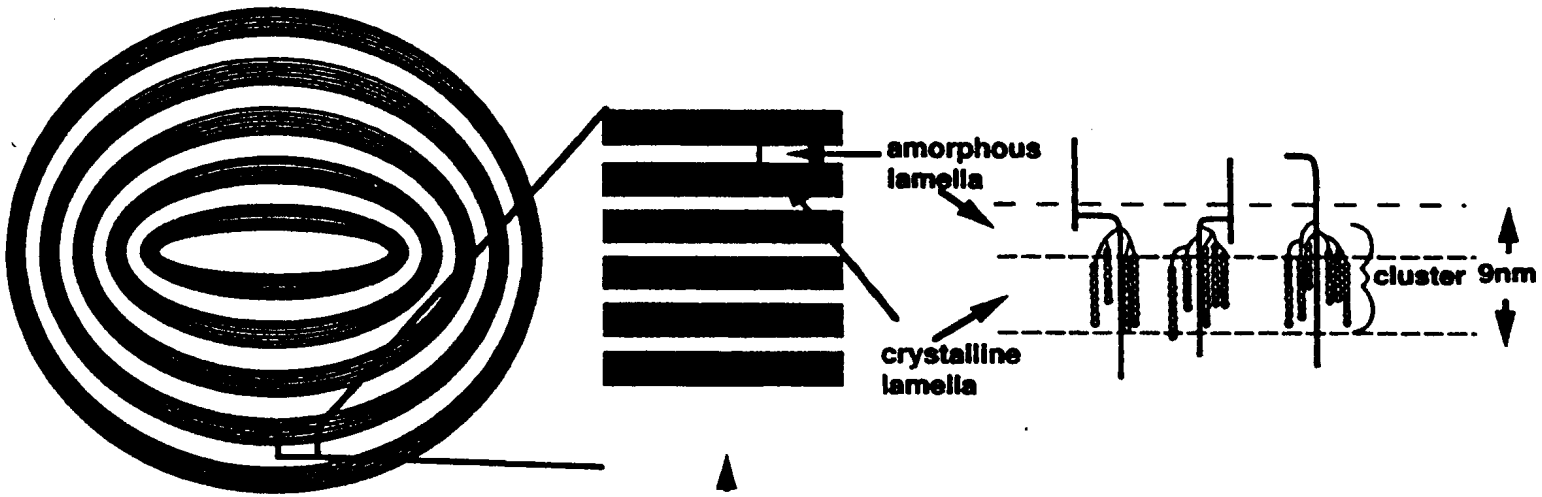
using better columns for SEC. However, SEC could not separate individual chains. 'A' and 'B_1' are considered to be the shortest chains having CL of 14-18, whereas the longer ones (B_2-B_4) have CL of 45-55. The proportion of short to long chains differs depending on the botanical source of starch (Hizukuri, 1985).

In general, the models proposed for the structure of amylopectin suggest that: (1) amylopectin is composed of compact parts of oriented chains (crystalline clusters) and, (2) branching points are not randomly distributed throughout the molecule (French, 1972; Robin *et al.*, 1974; Watanabe *et al.*, 1982).

Koizumi *et al.* (1987) reported that high-performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) was useful for the analysis of linear homoglucans with DP up to 50-60. Since then, this technique has been widely utilized to characterize CL distributions of amylopectins. Later, Wong and Jane (1995) recommended nitrate as a better eluting solvent for HPAEC-PAD than acetate.

Although the branch chains of amylopectin are drawn as straight lines for simplicity (**Fig. 2-5**), they actually exist as double helices (section **'C'** of **Fig. 2-6**) in the native state or in aged starch gels. During the synthesis of starch in amyloplast, the side-chains can remain parallel to the main backbone strand, allowing the formation of double helices and development of dense three-

Fig. 2-6 – A schematic model of the starch granule [In sections (a) and (b) dark areas represent crystalline regions and white areas represent amorphous regions, in section (c) crystalline lamellae contain amylopectin branches while inter-crystalline amorphous lamellae contain branch points of amylopectin. The cluster size is 9nm]. (Donald *et al.*, 1997 – with author's permission).



a) b) c)

dimensional structures. This phenomenon has been proved by conformational and molecular modeling of the branching point of amylopectin (Imberty and Perez, 1989). These molecules may have a diameter of about 100Å and a length of several thousand Å (Robin *et al.*, 1974; French, 1984). For most granular starches, the cluster model of amylopectin provides the basis for explaining the role of amylopectin as the dominant, partially crystalline polymer in the alternating crystalline arrays of double stranded helices and amorphous zones of dense branching and cluster interconnecting long chain segments (Biliaderis, 1998).

Normally, cereal starches have shorter chains in both long- and short-chain fractions and higher amounts of the short-chain fraction compared to those of tuber starches (Hizukuri, 1985, 1986). Experiments on conformation and molecular modeling have revealed that the side chains can remain parallel to the 'C' (main back-bone strand). This allows the formation of double helices and development of dense three-dimensional structures in the starch granule (Imberty and Perez, 1989).

Amylopectin (specially those from root and tuber starches) contain phosphate ester groups, mainly at C3 and C6 (Banks and Greenwood, 1975). For example, potato starch contains ~1 phosphate ester per every 200-500 anhydroglucose units associated with the amylopectin fraction (Nielsen *et al.*, 1994). Based on the ^{31}P-NMR spectra, Lim *et al.* (1994) showed that larger amounts of starch

phosphates were on C6 rather than on C3 in potato, sweet potato, lotus, arrowroot, green pea, and mung bean. Although the biosynthetic route of phosphorylation is not yet fully known, it is believed to be an integral part of starch synthesis (Nielsen *et al.*, 1994).

Amylopectin is unable to form stable complexes with iodine because of the short length of the unit chains. Only a small amount of iodine (<0.6%) is bound forming a red-brown color complex with a λ_{max} = 530-540nm (Biliaderis, 1998). With increasing external chain-length, the λ_{max} of the iodine-amylopectin complex also increases (Banks and Greenwood, 1975). Amylopectin does not form complexes with lipids. However, there was indirect evidence reported about such interactions by calorimetry (Eliasson and Ljunger, 1988).

2.4.2 Minor components of starch

Apart from amylose and amylopectin, native starches contain lipids, protein, and ash in varying (minute quantities compared to amylose and amylopectin) amounts. Lipids and proteins may occur either on the surface or inside the granules (Lillford and Morrison, 1997). These compounds are usually described as "minor components" because they are present in very low amounts compared to amylose and amylopectin. However, they impart a dramatic effect on physicochemical properties of starch. A protein content of less than 0.5% is typical, and for cereal starches, the typical lipid content is ~1%. However, the amounts of these minor components, particularly lipids and proteins, depend on

the efficiency of the washing procedure in starch isolation (Eliasson and Gudmundsson, 1996).

2.4.2.1 Intermediate material

Some starches, such as amylomaize and wrinkled pea, contain considerable amounts (*e g.* 29% in wrinkled pea) of intermediate material, *i e.*, starch molecules that cannot be categorized as either amylose or amylopectin (Banks and Greenwood, 1975; Shannon and Garwood, 1984; Colonna and Mercier, 1984; Hizukuri, 1996). These are heterogeneous in nature with respect to structure and molecular size. Banks and Greenwood (1975) reported that this intermediate polysaccharide fraction contains linear chains of DP 50-200 and slightly branched low molecular weight molecules with higher chain lengths than normal amylopectin. This intermediate material cannot be fractionated and characterized easily due to its structural heterogeneity. This fraction is reported to be highly unstable in solution and undergoes extensive retrogradation (Hizukuri, 1996).

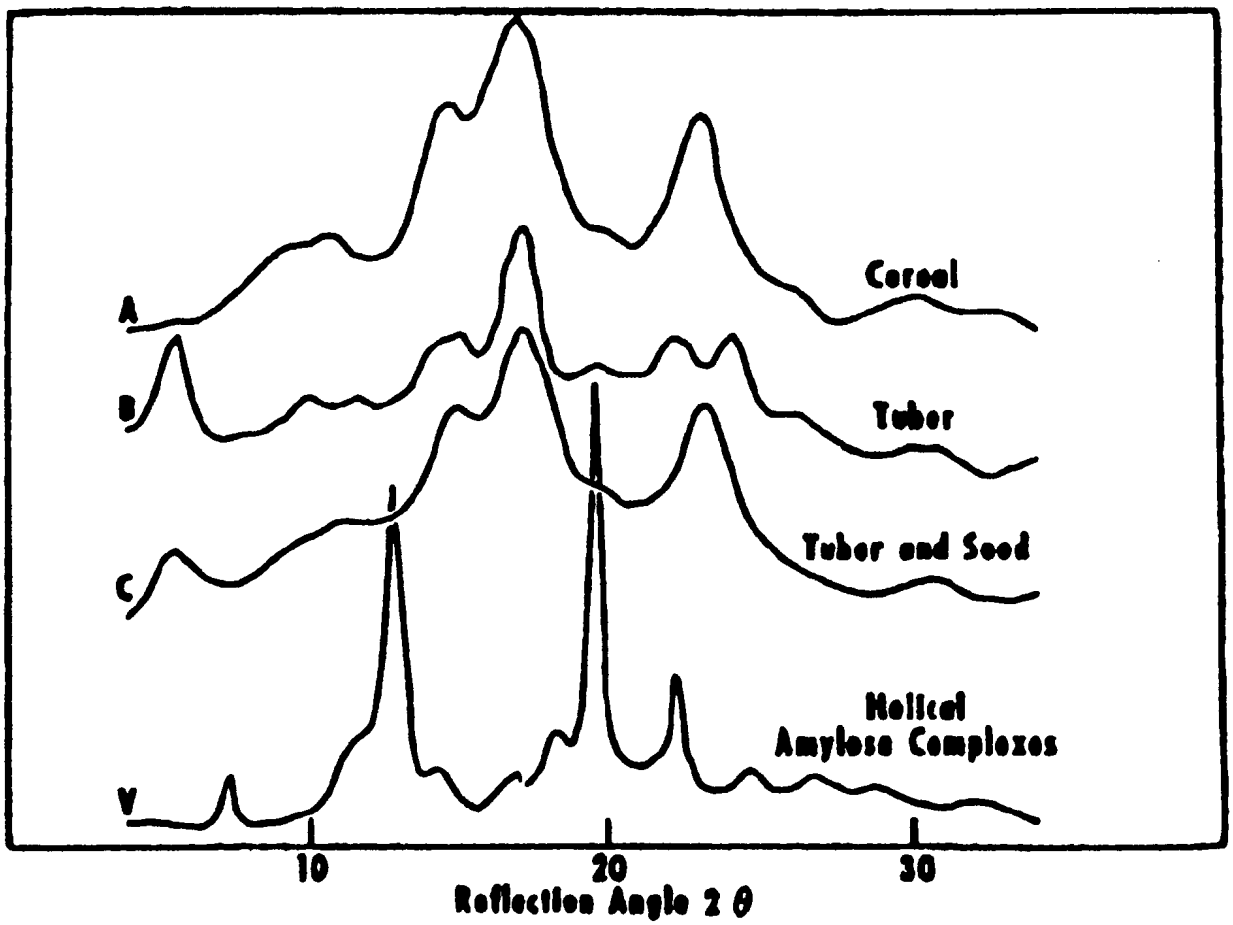
2.4.2.2 Lipids

Lipids associated with starch are found on the surface as well as inside the granule (Morrison, 1981; Vasanthan and Hoover, 1992b). The surface lipids are mainly triglycerides, and some amounts of free fatty acids, glycolipids and phospholipids are also found in isolated starch (Morrison, 1981; Galliard and Bowler, 1987). In most cereal starches, lysophospholipids are the main

components of lipid. The amount of free fatty acids varies from 1/3-1/2 of the total lipids in maize and rice (Morrison, 1998a). Waxy cereal starches contain only 1/10 of the amount of lipid found in their normal counterparts. For instance, amylomaize starch contains ~50% more lipids than normal maize with free fatty acids as the major component (Tan and Morrison, 1979). These lipids are in helical complex, which is called as 'V' (Verkleisterung) complex ('V' is based on the X-ray diffraction pattern of the amylose-lipid complex) with amylose in the granules (Zobel, 1988b) (**Fig. 2-3** and **Fig. 2-7**). Morrison *et al.* (1993) showed, by ^{13}C - cross polarization/magic angle spinning NMR spectroscopy, X-ray diffraction and DSC the existence of amylose-lipid complexes in native barley, maize, rice and oat starches. They further proved that amylose in cereal starches could occur as two fractions: (1) lipid-complexed amylose and (2) free amylose. Amylose-monoglyceride complexes of different CL exhibit the same wide-angle X-ray diffraction pattern (Biliaderis and Galloway, 1989; Hoover and Hadziyev, 1981). However, the intensity of the diffractogram depends on the monoglyceride chain length (Hoover and Hadziyev, 1981).

In contrast to Morrison *et al.* (1993), Zobel (1993) reported that naturally occurring 'V' complexes were not found in low amylose (<30%) starches. However, the absence of the 'V' pattern in native starches does not confirm the absence of amylose-lipid complexes in starch because the complex (in the crystalline domains) can be arranged in such a way that it could not be detected by wide-angle X-ray diffraction (Eliasson and Larsson, 1993).

Fig. 2-7 – X-ray diffraction patterns of 'A', 'B' and 'C' type starches, and 'V' amylose (Zobel, 1988b – with permission from John Wiley and Sons)


A
Cereal
B
Tuber
C
Tuber and Seed
V
Helical
Amylose Complexes
10
20
30
Reflection Angle 2 θ

2.4.2.3 Proteins

Normally, nitrogen in starch occurs in the form of protein (Lineback and Rasper, 1988), and the purity of starch can be expressed in terms of its protein content (purity increases with decreasing amounts of protein). The average protein contents of different starches have been reported as: 0.35% in maize, 0.4% in wheat, and 0.06% in potato (Swinkles, 1985a). Proteins could present in the form of either granule surface proteins or internal proteins/enzymes (Lowry *et al.*, 1981; Eliasson and Larsson, 1993). A part of the nitrogen could occur in association with starch lipids. Surface proteins can be readily extracted with dilute salt under mild conditions without disrupting the granules. However, the internal proteins are not released by dissociating agents (such as sodium dodecyl sulfate) until the granule is gelatinized by heating, because these proteins are buried within the matrix of the granules. The internal proteins are reported to be of higher molecular weight than those found on the granular surface (Lowry *et al.*, 1981).

2.4.3 Starch birefringence and crystallinity

Starch granules, when viewed under polarized light exhibit an optical birefringence pattern known as the 'Maltese cross'. The fact that starch is birefringent implies that there is a high degree of molecular order within the granule (Greenwood, 1979). Amylose and amylopectin are organized into a radially anistropic, semi-crystalline unit in the starch granules. This radial

anisotropy is known to be responsible for the distinctive Maltese cross (Blanshard, 1979).

The granules also exhibit an X-ray diffraction pattern showing that they have a certain degree of crystallinity and the granule is known to be comprised of crystalline and non-crystalline domains, and may have some transitional domains. The crystallinity of starch is essentially due to its amylopectin component (Banks and Greenwood, 1975; Blanshard, 1979; Zobel, 1988b; BeMiller, 1979; Biliaderis, 1998; Hizukuri, 1996) and the crystalline domains are believed to be constructed mainly by 'A' chains and outer 'B' chains of amylopectin (Hizukuri, 1996). The two main indications to prove this fact are: (1) waxy starches (with no amylose) give an X-ray pattern very similar to that of native starch, and (2) amylose can be readily leached from the granule leaving the structure intact (Greenwood, 1979). It is known that amylose and amylopectin molecules are arranged in granules to form alternative crystalline and amorphous regions commonly called as "growth rings". The short chains in the amylopectin molecule are organized into double helices, some of which then form crystalline lamellae or crystallites (Bertoft *et al.*, 1999; French, 1984) (**Fig. 2-6**). The crystalline order in the granules has been studied in depth by wide-angle X-ray powder diffraction methods on the basis of intensities and Bragg reflection angles (2θ). For a correct calculation of the total crystallinity, it is necessary to measure a wide range of angles, from at least 4 to 30° 2θ. It should also take into account the fact that the diffraction pattern contains a contribution from the

amorphous part (Wang *et al.*, 1998). The percent crystallinity of some native starches along with their corresponding amylose contents are shown in **Table – 2-6**.

Starch crystallinity is reported to be dependent on the moisture content of starch. It has been reported that the position and intensity of diffraction maxima were affected by hydration (Cleven *et al.*, 1978). Hydration causes the peaks of the diffractogram to increase in both intensity and sharpness. This may be due to the line up of the double helices along some common axis (Waigh *et al.*, 1997).

2.4.4 Amorphous region of the starch granule

Amylose in the starch granule is believed to be in a non-crystalline state, and found mainly in amorphous regions. Free amylose, lipid-complexed amylose and branch points of amylopectin are present in a mixture in the amorphous region. The conformation of the chains may be single helix or random coils. Some double helical chains may also be found in the amorphous region (Hizukuri, 1996). The amorphous part of the granule is less dense and, therefore, more susceptible to chemical and enzymatic modification (French, 1984; Hood and Mercier, 1978). Small (<1000Da) water-soluble molecules readily diffuse into the granule through the amorphous region (French, 1984).

Gallant *et al.* (1997) suggested that the crystalline and amorphous lamellae of amylopectin are organized into larger, spherical "blocklets" with diameters of 20-

Table 2-6 - Crystallinity and amylose contents of some native starches (Zobel, 1988a).

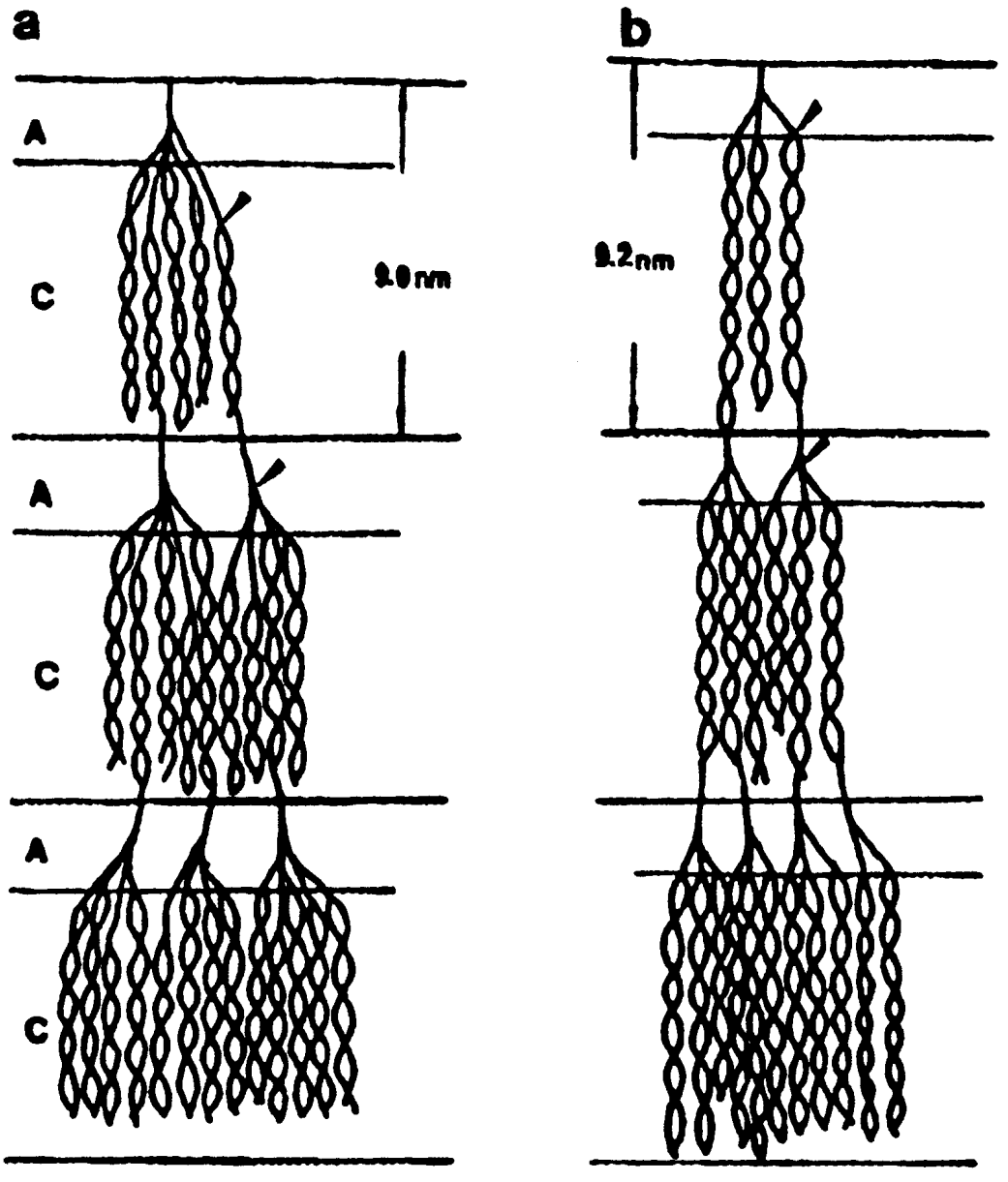
Starch	Crystallinity (%)	Amylose content (%)
'A' type starches		
Oat	33	23
Rye	34	26
Waxy rice	37	-
Rice	38	17
Corn	40	27
'B' type starches		
Amylomaize	15-22	55-75
Potato	28	22
'C' type starches		
Sweet potato	38	20
Tapioca	38	18

500nm and contain 5-50 number of amylopectin short DP clusters. With this type of organization, the amorphous material exists in four different regions within the granule as: (1) in the lamella (branch point of amylopectin), (2) among clusters of side chains within each lamella, (3) around each "blocklet" of side chain clusters, and (4) in radially arranged channels in granules through which amylose leaches during gelatinization. Jane *et al.* (1997), by analyzing Naegeli dextrins and debranched Naegeli dextrins of native cereal, root and tuber starches by HPAEC-ENZ-PAD and wide-angle X-ray diffraction, reported that in 'B' type starches, the amylopectin branch linkages were clustered in the amorphous region, whereas in 'A' type starches the branch linkages are located within the crystalline region (**Fig. 2-8**).

2.4.5 Polymorphic composition

Starches in their native state are semi-crystalline in nature and they are of three major polymorphic forms known as 'A', 'B' and 'C' (**Fig. 2-7**) (Zobel, 1988b). This classification is based on the X-ray diffraction patterns (first recorded by Katz and VanItallie, 1930) of the native starches, and wide-angle X-ray diffraction can be used to determine the type of polymorph in the starch. 'A' type starches are characterized by peaks (approximately) at 15°, 17°, 18°, 20° and 23° of 2θ angles while 'B' type starches are characterized by peaks at 5-6°, 15°, 17°, 20°, 22°, and 23° of 2θ angles in the diffractogram (Cheetham and Tao, 1998). The 'A' type starches are normally found in cereals and grains and the 'B' type starches are

Fig. 2-8 – Branching patterns of (a) waxy maize ('A' type), and (b) potato ('B' type) amylopectins. 'A' and 'C' in the figure stands for amorphous and crystalline regions respectively. The lengths between arrows stand for the lengths of internal 'B' chains (Jane *et al.*, 1997 – with permission from Elsevier Science).


a
A
C
9.0nm
A
C
A
C
b
9.2nm

found in tubers. A third 'C' type X-ray diffraction pattern, which is considered to be an intermediate of 'A' and 'B', is found in legume, root and banana starches (Sterling, 1968; Cairns *et al.*, 1997). 'C' type starches give diffractograms having characteristics intermediate to the 'A' and 'B' patterns, with prominent peaks at 2θ angles of 5-6°, 15°, 17°, 20°, and 23° (Cheetham and Tao, 1998). Bogracheva *et al.* (1998) showed that in 'C' type starches, the 'B' polymorphs are arranged centrally while the 'A' polymorphs are located peripherally within the granules using DSC, wide-angle X-ray diffraction, and ^{13}C NMR. They further reported that the arrangement of 'A' and 'B' polymorphs plays an important role in the swelling of the granules. The swelling and subsequent gelatinization begins in the center of the granule where 'B' polymorph is shown to be arranged.

It has been shown that the area under the small peak at 5-6° 2θ angle has a positive correlation with the amount of 'B' polymorph in the starch (Gernat *et al.*, 1990). This phenomenon has been successfully used to calculate the polymorphic composition in 'C' type starches by many researchers (Gernat *et al.*, 1990; Davydova *et al.*, 1995; Cairns *et al.*, 1997).

2.5 Properties of starch

2.5.1 Swelling and gelatinization

The swelling behavior of native starches varies depending on the botanical source and the thermal history of the sample. However, most starches follow the same general pattern of behavior. Starches are insoluble in cold water and swell

(reversible on drying) only to a limited extent. Cold water swelling of starch is an exothermic process. In this process, water penetrates into the amorphous regions and forms hydrogen bonds with hydroxyl groups on the starch molecules. The heat of hydration varies with, (1) the number of hydroxyl groups available for hydrogen bonding and (2) the amount of water used (Collison, 1968). The granules absorb more water upon heating of the starch-in-water suspension. First, the water absorption by amorphous regions facilitates the 'stripping' of the chains in the crystalline domains, which destabilizes neighboring crystallites (Biliaderis, 1990). These swollen granules contain entangled amylopectin molecules with amylose in between them. Upon further heating, the granules tend to swell to greater extents and the crystallites melt. This results in an increased motion of the molecules and amylose and amylopectin start to separate. These granules contain no crystalline regions since all the crystallites have melted completely during heating (Keetals *et al.*, 1996b; Levin and Slade, 1989). Tester and Morrison (1990) reported that swelling is primarily a property of amylopectin based on the observation that waxy barley starch swelled much more than did normal barley starch. Sasaki and Matsuki (1998) reported that the swelling power (measured as the weight of sedimented swollen granules per gram of dry starch) of starch correlated negatively with amylose content. Furthermore, they found that when amylopectin contained higher proportions of DP≥35 branches, the swelling power was also high. They also found that the gelatinization enthalpy and temperature are positively correlated with swelling power. The above findings suggested that the factors contributing to granular

crystallinity also influence the differences in starch swelling properties. Donovan and Mapes (1980) reported that when the amorphous parts of potato starch granules were removed by acid hydrolysis, the biphasic endotherm merged into a single endotherm (in DSC) at a higher temperature. This suggests that the amorphous region of the granule plays an essential role in the process of swelling by destabilizing the crystallites.

Jenkins and Donald (1998) suggested a slightly different theory for swelling and gelatinization of starch granules based on DSC, small angle and wide angle X-ray scattering and small angle neutron scattering. According to these authors, the gelatinization of starch in excess water is a two-stage process. In the first stage, the amorphous background region starts to swell extensively to take up water and this happens immediately prior to the gelatinization endotherm detected by DSC. In the second stage, the swelling process causes the disruption of crystallites in crystalline lamellae. They further suggested that this mechanism is common to all the starches regardless of the source.

Tester and Morrison (1990) concluded, by analyzing the swelling and gelatinization properties of native and defatted cereal starches, that the natural lipids in wheat starch caused a substantial suppression of swelling when they formed complexes with amylose. They further concluded that swelling was a property of amylopectin. However, Sasaki and Matsuki (1998) reported that there was no correlation between swelling power and starch lipid content.

Fredriksson *et al.* (1998) reported that amylose acts as a resistant to granular swelling.

When a suspension of unmodified starch granules in excess water is heated, a temperature is reached at which the hydrogen bonding forces holding together the constituent molecules of the granules are weakened. As a result, the granules can absorb far more water and swell. This will cause the polymer molecules (largely, but not exclusively, amylose) to leach out of the granules. Studies have shown that in potato (Cowie and Greenwood, 1957), barley (Banks *et al.*, 1959), amylomaize and pea (Banks and Greenwood, 1975), and wheat (Ghiasi *et al.,* 1982) starches, the molecules that leached out at low temperatures are lower molecular weight linear amylose and at higher temperatures higher molecular weight branched amyloses leach out. Amylose and amylopectin leach together at all temperatures in oat starches (Doublier *et al.,* 1987). With continued heating and shearing, the granules continue to swell, become fragile and eventually burst. The result is a discontinuous phase of swollen granules and/or granule fragments in a continuous phase of polymer solution. The properties of the paste are determined by: (1) the nature of the continuous phase and discontinuous phase, and (2) interactions between the two phases (Atwell *et al.*, 1988; BeMiller, 1979; Dengate, 1984; Levin and Slade, 1990). Studies have shown that the amylose content of the granule appears to decrease the intramolecular interactions between amylopectin units, resulting in an increased rate of breakdown of granular rings during gelatinization (Atkin *et al.*, 1998).

Starch gelatinization is an endothermic process and it can be monitored by thermal analysis. Calorimetric methods have been frequently applied to study phase transitions of aqueous suspensions of granular starch upon heating (Biliaderis, 1990; Cooke and Gidley, 1992). The gelatinization endotherm is attributed to the disordering and melting of amylopectin crystallites. This is different from endotherms due to the dissociation of crystallites formed during retrogradation or amylose-lipid complexes (Stute and Koniczny-Janda, 1983; Morrison, 1988b). Generally, DSC analysis gives a single endotherm for a starch-water system containing more than 60% water. However, when there is insufficient water available for gelatinization, some crystallites melt at a higher temperature due to hydration swelling of the amorphous part of the granule and its coupling with the crystallites. This results in a bi-phasic endotherm, where the nature of the second endotherm depends on the water content and thermal stability of the remaining crystallites (Donovan, 1979).

A higher gelatinization temperature indicates a higher proportion of longer chains in amylopectin. Longer chains would form longer double helices that would require a higher temperature to dissociate completely than that required for shorter double helices. The type of crystalline packing also affects the gelatinization temperature of starch granules (Yuan *et al.*, 1993). A broad endotherm might indicate a lack of homogeneity of ordered structures inside the granules. These granules might contain crystallites and double helices (which

are not in crystalline form) of widely varying length, resulting in a broad gelatinization temperature range (Yuan *et al.*, 1993). A bimodal endotherm (in the presence of sufficient water) shows the presence of two populations of granules or the existence of two types of molecules within the granules. The higher temperature endothermic peak would be consistent with the melting of longer crystallites formed by the longer 'A' chains and B_1 chains (Yuan *et al.*, 1993). Sahai and Jackson (1999) reported that the enthalpic transitions during DSC might not reflect melting of starch double helical structures, but they did not give an alternative theory.

2.5.2 Pasting and paste viscosity

Swelling and subsequent disruption or pasting of the starch granules can be readily followed by using a Brabender amylograph (**Fig. 2-9**). **Fig. 2-10** shows the pasting behavior of three genotypes of maize: normal maize, waxy maize (no amylose) and amylomaize (50% amylose). As the temperature is increased, granules swell and impinge on each other, resulting in an increased viscosity of the paste. This process continues until it reaches a peak viscosity, after which the granules lose the cohesive forces in their structure, become excessively weakened and collapse. This will result in a drop in the paste viscosity. Waxy starches swell rapidly to give a higher peak viscosity, but also breakdown rapidly due to their weak intermolecular forces on further cooking. The cooling cycle (from 95 to 50°C) measures the extent of 'set-back' due to the aggregation of amylose. Waxy starches contain very little amylose and, therefore, the set-back

Fig. 2-9 – Pasting cycle curve showing definitions of different pasting parameters (Dengate, 1984 – with permission from Butterworth [Publishers], Inc.).

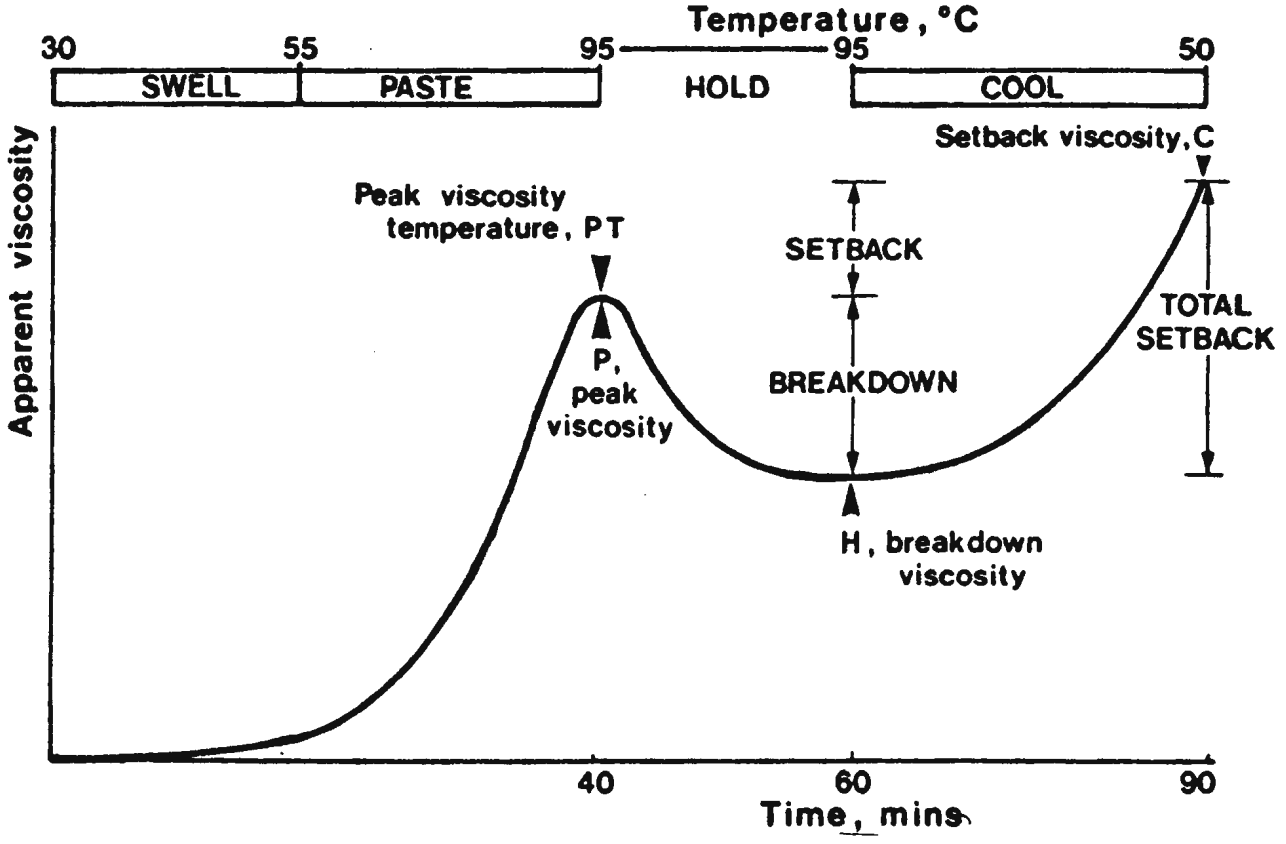
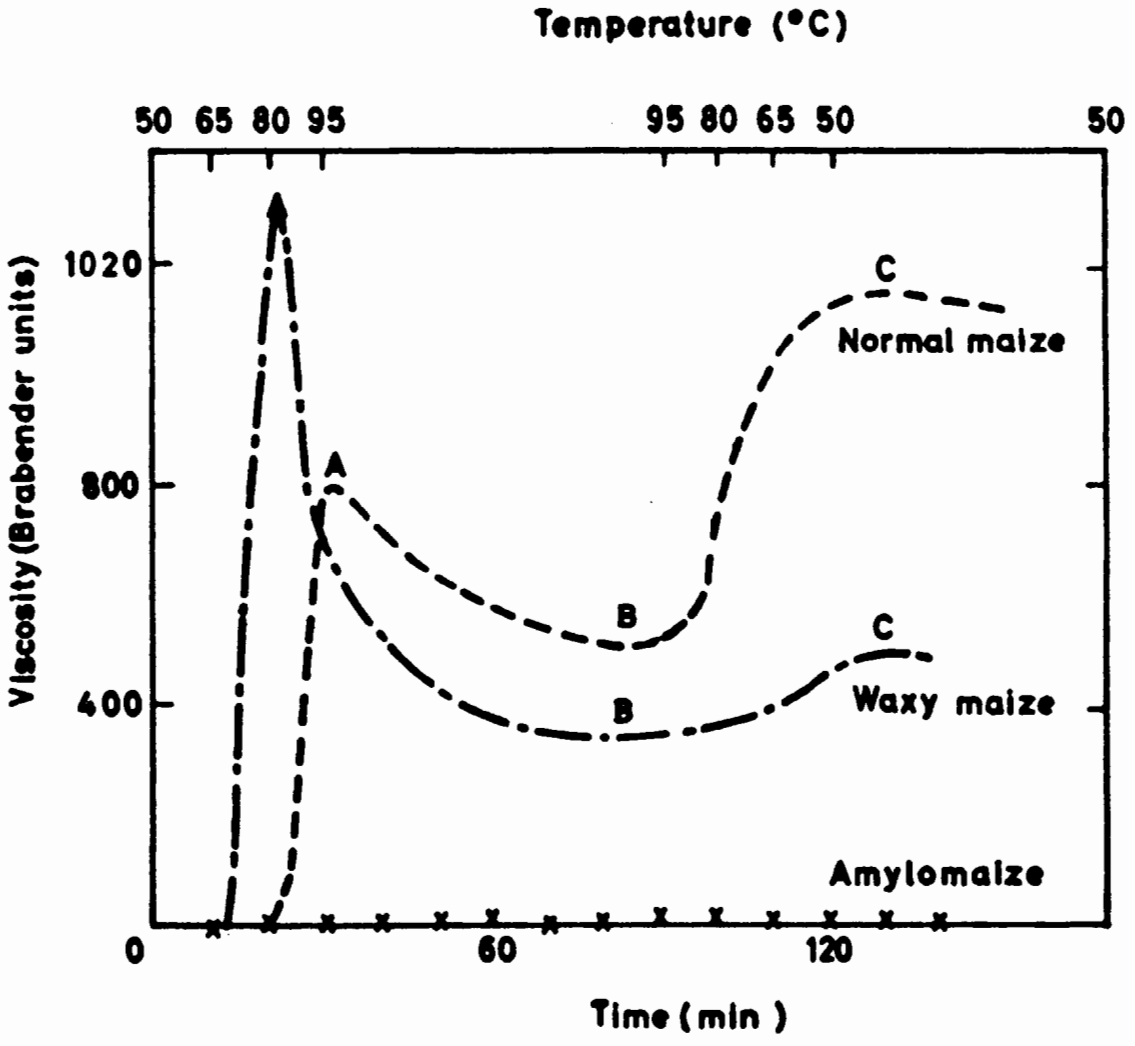

Temperature, °C
30
55
95
95
50
SWELL
PASTE
HOLD
COOL
Apparent viscosity
Setback viscosity, C
Peak viscosity temperature, PT
SETBACK
TOTAL SETBACK
P, peak viscosity
BREAKDOWN
H, breakdown viscosity
40
60
90
Time, mins

Fig. 2-10 – Brabender visco-amylograms of different maize starches (A = peak viscosity, B = extent of breakdown of paste structure after prolonged stirring at elevated temperature, and C = set-back [the development of aggregated structures on cooling]) (Greenwood, 1979 – with permission from Butterworth [Publishers], Inc.).


Temperature (°C)
50 65 80 95
95 80 65 50
50
1020
800
400
0
Viscosity (Brabender units)
A
B
C
Normal maize
B
C
Waxy maize
Amylomaize
60
120
Time (min)

is low. High amylose starches (*e g.* amylomaize in **Fig. 2-10**) are little affected and no swelling is apparent on the amylograph (Greenwood, 1979). Jane *et al.* (1999) reported that starch gelatinization temperature, enthalpy change, and pasting properties were determined by amylopectin branch chain lengths and distributions. They observed an increased gelatinization temperature with increasing branch chain length, and an increased pasting temperature and decreased peak viscosity with increasing amylose, lipid and phospholipid contents.

2.5.3 Retrogradation

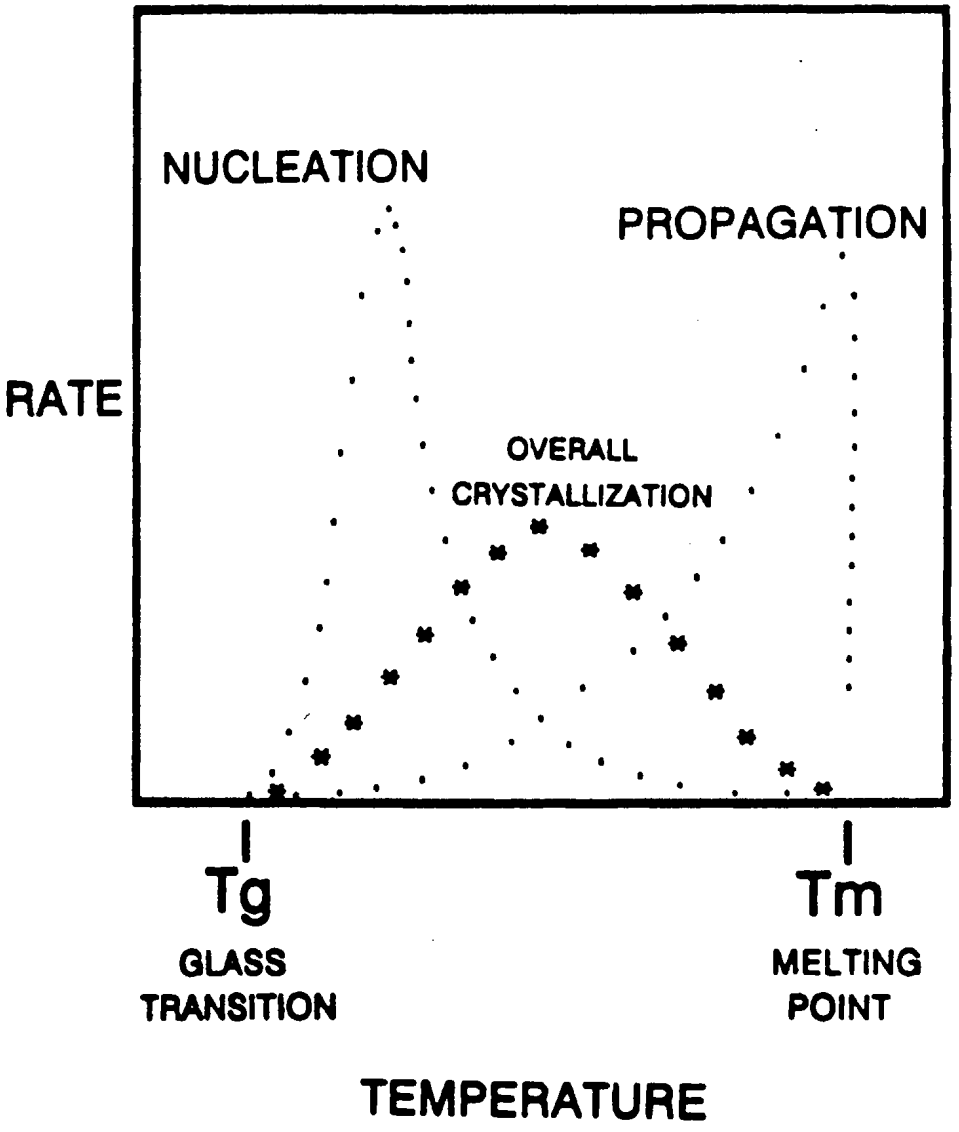
Retrogradation is generally defined as "a process, which occurs when the molecules comprising gelatinized starch begin to re-associate in an ordered structure". After gelatinization and pasting, the starch-water system is not in an equilibrium state and, therefore, undergoes structure transformation *via* chain aggregation and re-crystallization on storage. During the process, 'B' type crystallites are formed and the polymer and solvent phases tend to separate, resulting in 'syneresis' (Biliaderis, 1998). This re-association of the molecules leads to an increased firmness and staling of starchy foods (Jacobson and BeMiller, 1998). This process has a considerable industrial importance because of its major effects on texture and digestibility of starchy foods (Ring *et al.,* 1987).

Retrogradation proceeds in two stages (Miles *et al.*, 1985a). In the first stage, the rigidity and crystallization of starch gels develop quickly as a result of

amylose gelation and the increased mobility of the smaller size amylopectin fragments (Biliaderis, 1998; Zhang and Jackson, 1992). In the second stage, further crystallinity develops slowly in the amylopectin region (Biliaderis, 1998; Miles *et al.*, 1985a). Miles *et al.* (1985b) proposed a three-step mechanism to explain crystal development in amylose gelation based on the Avrami theory (Avrami, 1939, 1940). This mechanism involved: (1) nucleation [the formation of critical nuclei by initiation of ordered chain segments], (2) propagation [the growth of crystals from nuclei by molecular aggregation of ordered segments or 'packing' of double helices], and (3) maturation [crystal perfection by annealing of metastable crystallites]. Baik *et al.* (1997) reported that the overall recrystallization process of starch is mainly nucleation dependent, and it was highest at 4°C, whereas propagation was highest at 30°C and decreased over increasing temperatures. The recrystallization kinetics of starch retrogradation are shown in **Fig. 2-11**.

Hizukuri (1985) suggested that there is an irreversible phase separation into polymer rich and polymer deficient regions in amylose gelation. During subsequent retrogradation, the development of crystallinity, which is a slow process, occurs within the polymer rich phase. Gidley (1989) reported that the critical concentration of amylose for gelation to occur is nearly independent of chain length and around 1.0% (w/v). The rate of aggregation and the physical state of aggregated material are highly dependent on the CL of amylose in aqueous solutions (Miles *et al.*, 1985a). It has been reported that the gelation of

Fig. 2-11 - Recrystallization kinetics of starch, expressed in terms of crystallization rate as a function of temperature (Levin and Slade, 1990 - with permission from Van Nostrand Reinhold/John Wiley and Sons)


NUCLEATION
PROPAGATION
RATE
OVERALL
CRYSTALLIZATION
Tg
GLASS
TRANSITION
Tm
MELTING
POINT
TEMPERATURE

amylose is more pronounced when the molecules have a degree of polymerization (DP) of ~80 units and smaller and larger molecules were comparatively stable (Biliaderis, 1998). Gidley (1990) explained two types of amylose aggregations that occur in amylose gelation as: (1) alignment of short chains into sufficiently large aggregates that lead to precipitation, and (2) cross-linking of longer chains through alignment of chain segments with each other, leading to gelation. He further reported that although for many chain lengths (generally DP 100-1000) both processes occur, for shorter chain lengths (DP<40) cross-linking would not take place. The starch source and the amount of amylose solubilized during gelatinization have been shown to influence amylose gelation (Ellis and Ring, 1985). Temperature also has a significant influence on amylose retrogradation. Retrogradation of amylose does not take place at temperatures above 65°C (Miles *et al.,* 1985a). Lu *et al.* (1997) reported that different molecular weight fractions of amylose have different critical retrogradation temperatures.

Slade and Levin (1986) reported that starch retrogradation is mainly controlled by the non-equilibrium re-crystallization behavior of amylopectin. The distribution of branch chain lengths plays an important role in amylopectin retrogradation (Hizukuri, 1986; Kim *et al.,* 1997; Suzuki *et al.*, 1985; Ring *et al.*, 1987). Shi and Seib (1992) reported that the retrogradation of waxy starches is directly proportional to the molar fraction of DP 14-24 and inversely proportional to the mole fraction of DP 6-9. Amylopectin gels are comparatively weaker than

amylose gels and formed by pair-wise intermolecular associations, due to the helix formation between the outer 'A' chains of the neighboring amylopectin molecules (Cameron *et al.*, 1994). Keetals *et al.* (1996a) proposed two possible mechanisms to explain the increase in stiffness which takes place during re-crystallization of amylopectin: (1) formation of crystalline clusters along the glucan chains resulting in further stiffening of strands between entanglements, and (2) formation of cross-links between adjacent clusters. However, it was not clear which of the two plays a major role in the retrogradation of amylopectin. Unlike amylose, amylopectin gelation and retrogradation are thermo-reversible. This enables the use of DSC to follow these processes (Ring *et al.*, 1987).

A number of factors affect the rate of retrogradation. These include molecular ratio and structures of amylose and amylopectin, temperature (Hizukuri *et al.*, 1972), starch concentration, botanical source of the starch and the presence and the concentration of other ingredients (Leloup *et al.*, 1991; Bello-Perez and Paredes-Lopez, 1995; Lin and Czuchajowska, 1998; Orford *et al.*, 1987).

2.5.4 Starch hydrolysis

Either partial or total depolymerization of starch gives starch hydrolysates containing low-molecular weight carbohydrates. This process of depolymerization is attained by the action of either acids or enzymes. The shape and size of the granule, the proportion of amylose to amylopectin and the amount

of fat, protein and non-starch polysaccharides such as pentosans in starch influence starch hydrolysis (Swinkles, 1985b; Slominska, 1997).

2.5.4.1 Acid hydrolysis

Acids such as HCl and H_2SO_4 cause cleavage of the glycosidic linkages, altering the structure and properties of starch. The amorphous regions of the starch granule are relatively more susceptible to acid hydrolysis than the crystalline regions and all starches, regardless of the source, exhibit a two-stage hydrolysis pattern (Hoover, 2000). The faster stage corresponds to the degradation of amorphous parts of the starch granules, while in the second -relatively slower-stage, the crystalline material is slowly degraded (Robin *et al.*, 1974; Kainuma and French, 1972). Transmission electron microscopic investigations of acid hydrolyzed starches have proved the existence of a preferential etching of amorphous growth rings from normal and waxy maize starches treated with 7% HCl at room temperature for 35 days (Mussulmann and Wagoner, 1968). The slower rate of hydrolysis of the crystalline part of the granule is due to the restriction of the conformational change ('chair' to 'half-chair') of the D-glucopyranosyl unit by the immobilization effect of the crystalline structure (French, 1984).

Information on the hydrolysis kinetics of native starch by 2.2*N* HCl is widely used to study the structure of the granules (Colonna *et al.*, 1982; Hoover and Vasanthan, 1992; Muhr *et al.*, 1984). The solubilization pattern and the extent of

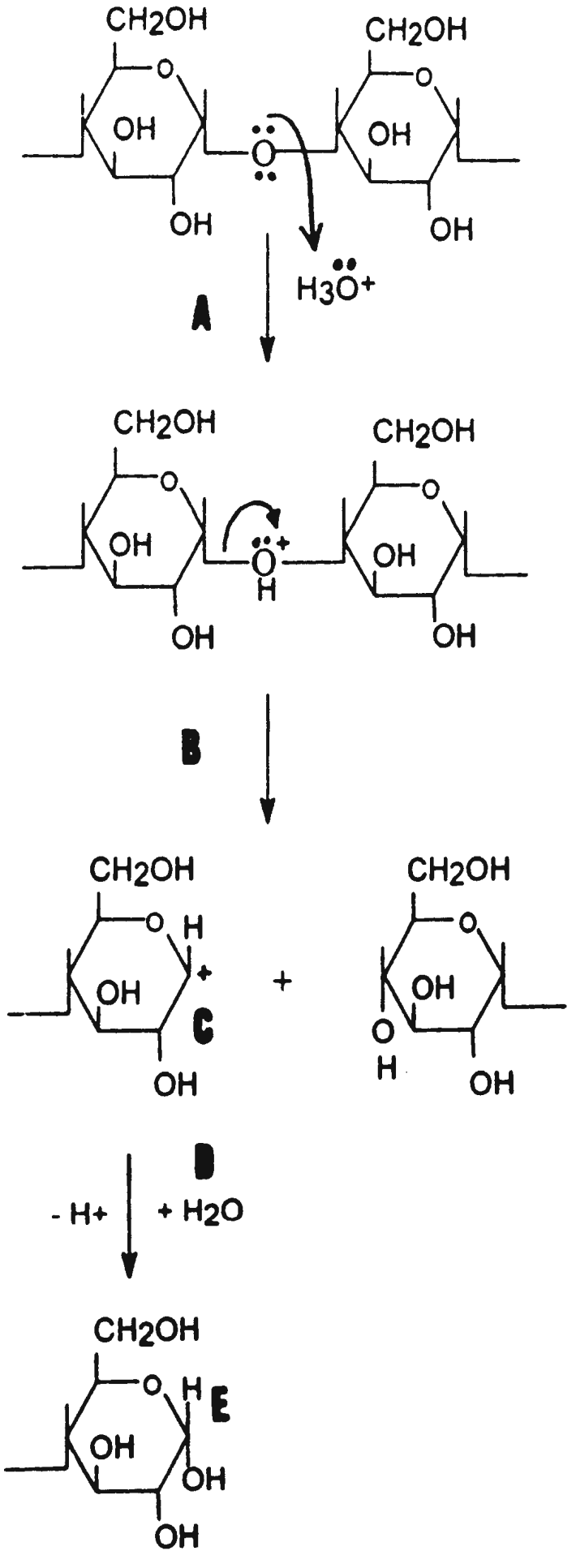
hydrolysis give indirect information about the size of the amorphous and crystalline regions and the degree of helical packing in the crystalline region of the starch granule. The differences in the rates and extents of hydrolysis among starches are known to be due to differences in granular size, the nature of the starch molecule interactions within the amorphous and crystalline domains, and composition (*i e.,* amylose content, amount of lipid-complexed amylose and extent of phosphorylation) (Hoover, 2000).

In acid hydrolysis of starch (**Fig. 2-12**), protons carry out an electrophillic attack on the oxygen atom of the glucosidic bonds in molecules. This is known to be a unilateral electrophillic attack. In the second step, the electrons in one of the carbon-oxygen bonds move onto the oxygen atom generating an unstable high-energy carbocation intermediate. This intermediate then reacts with water to regenerate a hydroxyl group (Zherebtsov *et al.*, 1995; Hoover, 2000). Acid catalyzed hydrolysis requires aggressive conditions such as low pH and higher temperatures. These conditions may cause a partial decomposition of the glucose produced (Zherebtsov *et al.*, 1995).

2.5.4.2 Starch digestibility by α-amylase

Digestibility of starch by porcine pancreatic α-amylase has been studied by various researchers (Holm *et al.*, 1983; Seneviratne and Biliaderis, 1991, Cone and Wolters, 1990). Unlike many other enzymes (*e g.* β-amylase, pullulanase),

Fig. 2-12 – The mechanism of acid hydrolysis of starch (Hoover, 2000 – with author's permission).


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

α-amylase can attack native starch granules, and can solubilize both amorphous and crystalline regions without any preference (Leach and Schoch, 1961; Lauro *et al.*, 1999). Porcine pancreatic α-amylases has an active site with five D-glucose sub-sites (Robyt and French, 1970) and a catalytic site, which consists of a carboxylate anion (nucleophile) and imidazolium cation (electrophile) (Hoover and Sosulski, 1985). The hydrolytic action of porcine pancreatic α-amylase on starch molecules occurs by a multiple attack mechanism (Robyt and French, 1970), in which the enzyme can catalyze the hydrolysis of several bonds before it dissociates. The direction of this enzyme attack is from the reducing to the non-reducing end of the starch molecule (Robyt and French, 1970). Being endo-enzymes, α-amylases catalyze random hydrolysis of the starch components and the action is restricted to α(1-4) glycosidic linkages, which are either non-terminal or are not in the immediate vicinity of an α(1-6) interchain linkage. From amylose, the end products are maltose (~90%) with small amounts of glucose and/or maltotriose. From amylopectin, the same end products are produced together with branched oligosaccharides (α-dextrins), which contain original inter-chain linkages. Therefore, from starch, the end products are largely maltose, with smaller amounts of glucose, maltotriose and α-dextrins (Manners, 1979).

The enzyme hydrolysis of α-D glucose linkages is involved in an enzyme induced ring distortion of one of the D-glucosyl residues (from $^4C_1$ 'chair' conformation to 'half-chair' conformation). The ring distortion decreases the enthalpy of

activation and the susceptibility of the glucosyl residues to nucleophilic attack by functional groups of α-amylase (Thoma, 1968).

The *in vitro* digestibility of starch by porcine pancreatic varies according to: (1) starch crystallinity (Hoover and Sosulski, 1985; Ring *et al.*, 1988), (2) amylose/amylopectin ratio of the starch (Atkins and Kennedy, 1985), (3) granular size (Cone and Wolters, 1990; Franco and Ciacco., 1992), (4) starch-lipid interactions (Seneviratne and Biliaderis, 1991; Holm *et al.*, 1983), (5) starch-protein interactions (Wursh *et al.,* 1986), (6) starch retrogradation (Jane and Robyt, 1984; Kim *et al.*, 1997), and (7) starch modification (Wootton and Chaudhry, 1981; Hoover *et al.*, 1993; Hoover and Manuel,1996b). Guraya *et al.* (1997) reported that the *in vitro* digestion of starch by α-amylase was reduced by the complexation of amylose and amylopectin with fatty acids of different chain lengths. They further reported that long chain, saturated emulsifiers reduced enzymatic digestibility more than the short chain saturated and unsaturated emulsifiers.

3 Materials and methods

3.1 Materials

Field pea (*Pisum sativum* L.) cultivars (Carneval, Carrera, Grande and Keoma) were grown on experimental plots (under identical environmental and soil conditions) at the Morden Research Center, Agriculture and Agri-Food Canada, Morden, MB. Crystalline porcine pancreatic α-amylase (EC 3.2.1.1, type 1A), α-amylase from sweet potato (EC 3.2.1.2) and amyloglucosidase from *Rhizopus* mold (EC 3.2.1.3) were purchased from Sigma Chemical Co., (St. Louis, MO, USA). Isoamylase (EC 3.2.1.68) from *Pseudomonas amyloderamosa* and maltopentose were purchased from Hayashibara Biochemical Laboratories Inc., (Okayama, Japan). Maltotriose, maltotetrose, maltohexose and maltoheptose (for HPAEC-ENZ-PAD analysis of amylopectin) were purchased from Aldrich Chemical Co., (Milwaukee, WI, USA). Nucleosil 300-10 silica gels were purchased from Altech (Deerfield, IL, USA). Potato starch and waxy corn starch were gifts from National Starch and Chemical Co., Bridgewater, NJ, USA. All other chemicals and solvents were of ACS certified grade. Solvents were glass distilled before use.

3.2 Methods

3.2.1 Starch isolation

Three lots of field pea seeds were taken representing whole samples from the experimental plots of each cultivar. Starch was extracted from each lot using the

following procedure (Hoover and Sosulski, 1985). Sub-sub samples from each sub sample of starch from each cultivar were taken for the experiments.

Starch was isolated from field pea seeds by the procedure of Hoover and Sosulski (1985). Seeds (200g) were steeped in 300mL of 0.01% (w/v) sodium metabisulfite for 12h at 40°C. The swollen seeds were thoroughly washed with water and homogenized in a Waring Commercial blender (Dynamics Corporation of America, New Hartford, CT, USA) for 90sec. The homogenate was then filtered (under vacuum) through a double-layer cheesecloth. The filtrate was collected and left to settle for 2h. The sediment was suspended in excess 0.2% (w/v) NaOH and after standing for 12h the supernatant (water) was removed. The sedimentation procedure was repeated thrice. The final sediment was suspended in distilled water and passed through a 70μm polyproplylene filter cloth under vacuum. The filtrate was then passed through a 20μm polyproplylene filter cloth under vacuum. The residue was thoroughly washed with distilled water on a Buchner funnel under vacuum and re-suspended in water and neutralized to pH 7.0 with HCl. The slurry was filtered through a double-layer of Whatman No. 4 filter paper and the filter cake was oven dried at 28-30°C for 24h in a Fisher Isotemp 615G forced air oven (Fisher Scientific, Nepean, ON, Canada). The dried starch cake was carefully crushed and sieved to obtain a free flowing powder.

3.2.2 Granule morphology

Granule morphology of native starches was studied by scanning electron microscopy. Starch samples were mounted on circular aluminum stubs with double sticky tape and then coated with gold (20nm) and examined and photographed in a Hitachi - S 570 scanning electron microscope (Nissei Sangyo Inc., Rexdale, ON, Canada) at an accelerating potential of 20 kV.

3.2.3 Proximate analyses

3.2.3.1 Moisture content

Quantitative estimation of moisture was performed according to standard AACC (American Association of Cereal Chemists, 1984) procedures. Pre-weighed (4-5g) starch samples were dried in a forced air oven (Fisher Isotemp 615G, Fisher Scientific, Nepean, ON, Canada) at 130°C for 1h. The samples were then removed and cooled in a desiccator. The moisture content was calculated as a percentage weight loss of the sample.

3.2.3.2 Ash content

Pre-weighed (5±0.01g) samples were transferred into clean, dry porcelain ashing-crucibles, and ignited over a flame until thoroughly carbonized. Then they were placed in a pre-heated (525°C) muffle furnace (Lab Heat – Blue M model M30A-1C, Blue M Electric Co., Blue Island, IL, USA) and left until the sample was free from carbonaceous matter (~5h). The sample was cooled to room temperature in a desiccator and weighed. The ash content was calculated as the

percentage weight of the residue (American Association of Cereal Chemists, 1984).

3.2.3.3 Nitrogen content

Nitrogen content was determined by the Micro-Kjeldahl method. Samples (0.3g, db) were weighed on nitrogen free papers and placed in digestion tubes on a Buchi 430 digester (Buchi Laboratorimus-Technik AG, Flawill/Schweiz, Switzerland). The catalyst (two Kjeltabs M pellets) and 20mL of concentrated sulfuric acid were added and the samples were digested until a clear yellow solution was obtained. The digested samples were then cooled, diluted with 50mL of distilled water, 100mL of 40% (w/v) NaOH was added, and the released ammonia was steam distilled into 50mL of 4% (w/v) boric acid (H_3BO_3) containing 12 drops of end-point indicator (N-point indicator, EM Science, NJ, USA) using a Buchi 321 distillation unit until 150mL of distillate was collected. The amount of ammonia in the distillate was determined by titrating against 0.05N Sulfuric acid (American Association of Cereal Chemists, 1984). Percentage nitrogen was calculated as:

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

3.2.3.4 Lipid content

Different categories of lipids were extracted from starch by the following procedures and the amounts of extracted lipids were expressed as percentage weight of lipid per initial weight of starch.

3.2.3.4.1 Surface lipids

Surface lipids were extracted at room temperature (25-27°C) by mixing starch (5g, db) with 100mL of 2:1 (v/v) chloroform - methanol under vigorous agitation in a wrist action shaker for 1h. The solution was then filtered (Whatman No. 4 filter paper) into a round bottom flask and the residue was washed thoroughly with small amounts of chloroform - methanol solution. Then solution was evaporated to dryness using a rotary evaporator (Rotavapor – R110, Buchi Laboratorimus – Technik AG, Flawill/Schweiz, Switzerland) (method was slightly modified from Goshima *et al.*, 1985). The crude lipid extracts were purified by the method of Bligh and Dyer (1959) before quantification. The starch residue was saved for bound lipid extraction.

3.2.3.4.1.1 Bligh and Dyer (1959) method of lipid purification

The crude lipids from the above extracts were purified by extraction with chloroform - methanol - water (1:2:0.8 v/v/v) and by forming a biphasic system (chloroform - methanol - water, 1:1:0.9, v/v/v) by the addition of chloroform and water at room temperature in a separatory funnel. The chloroform layer was then diluted with benzene and brought to dryness on a rotary evaporator (Rotavapor –

R110, Buchi Laboratorimus – Technik AG, Flawill/Schweiz, Switzerland) followed by drying at 60°C for 1h in a forced air oven. The dried lipids were cooled to room temperature in a desiccator.

3.2.3.4.2 Bound lipids

Bound lipids were extracted using the residue left from surface lipid extraction. The residue was refluxed with 3:1 (v/v) 1-propanol - water in a soxhlet apparatus at 85°C for 7h (Vasanthan and Hoover, 1992b). The solvent was evaporated using a rotary evaporator (Rotavapor – R110, Buchi Laboratorimus – Technik AG, Flawill/Schweiz, Switzerland). Crude lipid extract were purified by the Bligh and Dyer (1959) method before quantification.

3.2.3.4.3 Total lipids

Total starch lipids were determined by hydrolyzing starch (2g, db) with 25mL of 24% (v/v) HCl at 70-80°C for 30min and extracting the hydrolysate three times with n-hexane. The mixture was evaporated to dryness in a rotary evaporator (Rotavapor – R110, Buchi Laboratorimus – Technik AG, Flawill/Schweiz, Switzerland) (Vasanthan and Hoover, 1992b). The crude lipid extract were purified by Bligh and Dyer (1959) method before quantification.

3.2.3.5 Amylose content

Apparent and total amylose contents were determined as described by Hoover and Ratnayake (2001).

3.2.3.5.1 Apparent amylose

Starch (20mg, db) was accurately weighed into a round bottom screw cap tube fitted with a Teflon® faced rubber liner in the cap. This was followed by the addition of 8mL of 90% dimethylsulfoxide (DMSO). The contents were vigorously mixed for 2min using a vortex mixer followed by heating in a water bath (PolyScience, Model 2L-M, PolyScience, Niles, IL, USA) at 85°C for 15min with intermittent shaking. The tubes were then allowed to cool to room temperature (~45min) and diluted to 25mL in a volumetric flask. One milliliter of the diluted solution was mixed with water (40mL), 5mL of I_2/KI solution (0.0025M I_2 and 0.0065M KI mixture) was added, and the final volume was adjusted to 50mL in a volumetric flask. After 15min (for color development), the absorbance was measured at 600nm using a UV-visible spectrophotometer (LKB Novaspec – 4049 spectrophotometer, LKB Biochrom Ltd., Cambridge, England). In order to correct for over-estimation of amylose content (due to complex formation between I_2 and long outer branches of amylopectin), amylose content was calculated from a standard curve prepared using mixtures of pure potato amylose and amylopectin [over the range 0-100% amylose and 100-0% amylopectin] (**Fig. 1-A** in **Appendix 1**).

3.2.3.5.2 Total amylose

Starch samples were defatted by extracting in a Soxhlet extractor (85°C) with 3:1 (v/v) n-propanol - water for 7h prior to the determination of total amylose content by the above procedure.

3.2.4 Starch damage

Starch damage was estimated following the standard AACC (American Association of Cereal Chemists, 1984) procedures. Starch samples (1g, db) were digested with fungal α-amylase from *Aspergillus oryzae* (12,500 units) having a specific activity of 50-100 units/mg in a water bath (PolyScience waterbath, PolyScience, Niles, IL, USA) at 30°C for 15min. At the end of the incubation, the enzyme action was terminated by adding 3.68*N* sulfuric acid (3mL) and 12% (w/v) sodium tungstate ($Na_2WO_4.2H_2O$) (2mL), respectively. The mixtures were allowed to stand for 2min and then filtered through Whatman No. 4 filter paper. Aliquots (5mL) of the filtrate were mixed with 2mL of chilled 3,5 dinitrosalycylic (DNS) acid and then diluted to 4mL with distilled water. The diluted samples were heated in a boiling water bath for 5min. The reaction mixture was chilled using an ice bath and diluted with 8mL of distilled water. The absorbance was measured at 540nm against a reagent blank (Bruner, 1964) [the details of the procedure are explained in the section 3.2.5.2.2.1]. A calibration curve (**Fig. 1-B** in **Appendix - 1**) was established with maltose (to calculate the maltose equivalents in the digest) and the percentage starch damage was calculated using the following equation.

$$\%\text{Starch damage} = [M \times 1.64] / [W \times 1.05] \times 100$$

where M = mg maltose equivalents in the digest, W = mg (db) of starch, 1.05 = molecular weight conversion of starch to maltose, and 1.64 = the reciprocal of the

mean percentage maltose yield from gelatinized starch (an empirical factor which assumes that under the conditions of the experiment, the maximum degree of hydrolysis is 61%).

3.2.5 Starch fractionation

Amylose and amylopectin were extracted from the field pea starches utilizing the aqueous leaching procedure described by Montgomery and Senti (1958).

3.2.5.1 Amylose

Defatted (3:1, v/v, *n*-propanol - water) field pea starch (40g, db) was extracted at 2% concentration by adding starch slurry to water at 98°C and maintaining this temperature for 15min while stirring the solution. Phosphate buffer (a 0.2*M* NaH_2PO_4, 45mL and 0.2*M* Na_2HPO_4, 55mL mixture was diluted to 200mL with distilled water) was used to maintain the pH of the solution between 6.0-6.3. The solution was cooled rapidly to room temperature (25-27°C), and then centrifuged (IEC-Centra MP-4, International Equipment Co., Needham, MA, USA) at 10000rpm for 20min in order to separate the supernatant and the gel-like material (which settled to the bottom of the centrifuge tube). The supernatant was saved and the gel was re-extracted with hot distilled water and phosphate buffer. The supernatant obtained after centrifugation was combined with the first supernatant and the gel was re-extracted a third time. The supernatant from the third extraction was discarded and the gel was saved for the isolation of amylopectin.

Amylose was isolated from the supernatant obtained after centrifugation. To the supernatant, 1-butanol (250mL) was added and stirred for 5h. The supernatant was decanted after centrifugation and the butanol-amylose complex was collected. The complex was recrystallized by adding it to 1L of boiling distilled water containing 100mL of 1-butanol. Stirring was continued until the solution became clear. The hot solution was filtered with suction and cooled slowly with stirring. An additional 150mL of 1-butanol was added, and stirring continued for 16h. The complexation process was repeated three times. The complex was then mixed with acetone in a blender (Waring Commercial blender, Dynamics Corporation of America, New Hartford, CT, USA) and filtered (Whatman No. 4). The filtered complex was resuspended in the blender with 95% ethanol and recovered again by filtration, treated with 99% ethanol in the blender, recovered by filtration and washed with diethylether. The recovered amylose was allowed to air-dry for 24h and then vacuum-dried at 40°C overnight (Montgomery and Senti, 1958).

3.2.5.2 Amylopectin

Amylopectin was precipitated from the gel after mixing with methanol (100mL) in a Waring blender for 45sec. The resulting white precipitate was allowed to settle. The methanol was decanted and fresh methanol (100mL) was added, followed by further blending (45sec.). The mixture was filtered under suction, and the precipitate was collected, again mixed with methanol in the blender, and

recovered by filtration. The precipitated amylopectin was air-dried for 24h and vacuum-dried at 40°C overnight (Montgomery and Senti, 1958).

3.2.6 Branch chain length distribution of Amylopectin

Isolated amylopectins were debranched using isoamylase according to the procedure of Jane and Chen (1992). Branch chain-lengths were obtained by using a high performance anion exchange chromatograph with a post column amyloglucosidase reactor and a pulsed amperometric detector (Wong and Jane, 1997) (**Fig. 2-A** in **Appendix 2**). The separation of debranched samples was carried out using a PA-100 anion exchange analytical column, a PA-100 guard column (Dionex, Sunnyvale, CA, USA), and an AS 40 automated sampler. The mobile phase used for separation consisted of eluent A (100mM NaOH) and eluent B (100mM NaOH with 300mM $NaNO_3$) with a flow rate of 0.5mL/min. The separation gradient was programmed as follows: 0-5min, 99% A and 1% B; 5-30min, linear gradient to 8% B; 30-150 min, linear gradient to 30% B; 150-200min, linear gradient to 45% B. The eluent degas module (Dionex, Sunnyvale, CA, USA) was set at a system pressure of 7psi (never exceeding 10psi). Pump A (Dionex DX-300 standard bore gradient pump), which delivered the gradient for sample separation, was operated at 600psi pressure (never exceeding 5000psi). Pump B (Dionex DX-300 micro bore gradient pump), which delivered 0.5N acetate buffer for pH adjustment, was operated at a minimum pressure of 600psi (not exceeding 5000psi). Pump C (Dionex pneumatic pump), which delivered 750mM NaOH solution for pH adjustment, was operated at 47psi. The entire

system was operated using an AI-450 software interface with an IBM compatible computer.

3.2.6.1 Amyloglucosidase column preparation

The enzyme column was prepared by vacuum slurry packing of the enzyme-immobilized support into a 2mm (i.d.) x 23 mm column. The enzyme column was then connected to the buffer pump and packed by the buffer solution at a flow rate of 0.5mL/min. The vacuum packing and buffer pump packing were repeated until the column was filled to the top.

3.2.7 Determination of amylose structure

3.2.7.1 Determination of the purity of isolated amylose by gel permeation chromatography (GPC)

Gel permeation chromatography on isolated field pea amylose was done by McPherson and Jane (1999) method with modifications. Five milliliters of the sample solution containing 15mg of isolated field pea amylose and 0.5mg of glucose (as marker) was injected into an Econo-Column® (1.5x100cm, i.d x l) (Bio-Rad Laboratories, Richmond, CA, USA) packed with Sepharose CL-2B (Sigma Chemical Co., St. Louis, MO, USA). 0.02% NaCl was used to elute the sample at 30ml/h flow rate. Fractions of 4.8mL were collected and analyzed for iodine affinity (0.0025*M* I_2/0.0065*M* KI solution) and total carbohydrate (Dubios *et al.*, 1956).

3.2.7.1.1 Iodine affinity

An aliquot of 0.250mL from each fraction was mixed with 5mL of I_2/KI (0.0025*M* I_2 in 0.0065*M* KI solution). The reaction mixture was allowed to stand for 15min at room temperature (25°C) for color development, and the absorbance was then measured using a LKB Novaspec – 4049 spectrophotometer (LKB Biochrom Ltd., Cambridge, England) at 600nm.

3.2.7.1.2 Determination of total carbohydrate (Dubios *et al.*, 1956)

Fractions (0.125mL) were diluted to 2mL using distilled water and the amount of total carbohydrate of each diluted fraction was analyzed as follows. One milliliter of 5% (w/v) phenol solution was added to 2mL of the sample solution in a test tube. Then, 5mL of conc. H_2SO_4 was added directly onto the sample liquid surface and the mixture was allowed to stand for 10min. The test tubes were transferred into a 30°C water bath after mixing. After 15min, the absorbance was taken against a reagent blank at 490nm. A standard series was prepared with known amounts of maltose (Fisher Scientific, Fair Lawn, NJ, USA) (**Fig. 1-C** in **Appendix 1**).

3.2.7.2 Degree of polymerization (DP) of amylose

Isolated amylose (0.10g) was completely dissolved in 10mL of dimethylsulfoxide by heating at 60°C in a water bath with occasional mixing. The resulting solution was divided into two equal volumes and the degree of polymerization (DP) was calculated using the following equation (Jane and Robyt, 1984).

$$DP = \frac{\text{Total carbohydrate } (\mu g)}{\text{Reducing sugar (as } \mu g \text{ of maltose)}} \times 2$$

Total carbohydrates and total reducing power were calculated according to the procedures outlined by Dubios *et al.* (1956) and Bruner (1964), respectively.

3.2.7.2.1 Determination of total reducing sugars (Bruner, 1964)

In the determination of total reducing sugars, 1mL of the sample solution was taken into a screw cap tube and the volume was adjusted to 2mL using distilled water. Then, 2mL of 3,5 dinitrosalycylic acid (DNS) solution [20g of DNS was dissolved in 700mL of 1*M* NaOH. The mixture was stirred well to dissolve DNS and then diluted to 1L with distilled water and filtered through a medium porosity fitted glass-filter (The reagent was stored in a dark bottle under refrigeration until used)] was added. The mixture was heated in a boiling water-bath for 5min for color development. The tubes were then cooled in an ice-bath for 10min, and then distilled water was added to make the volume up to 12mL. The absorbance was taken at 25°C using a UV-visible spectrophotometer (LKB Novaspec – 4049 spectrophotometer (LKB Biochrom Ltd., Cambridge, England) against a reagent blank. A standard series was prepared with known amounts of maltose (Fisher Scientific, Fair Lawn, NJ, USA) (**Fig. 1-B** in **Appendix 1**).

3.2.8 X-ray diffraction

X-ray diffractograms were obtained with a Rigaku RU 200R X-ray diffractometer (Rigaku-Denki Co., Tokyo, Japan). The operating conditions were as follows: target voltage 40kV, target current – 100mA, aging time – 5min, scanning range – 3-35°, scan speed – 2.000°/min, step time – 4.5sec, divergence slit width – 1.00, scatter slit width – 1.00 and receiving slit width – 0.60.

3.2.8.1 Determination of relative crystallinity

Crystallinity of the starches was calculated using the method of Nara *et al.* (1978) (**Fig. 2-B** in **Appendix 2**), using the peak-fitting software Origin – Version 6.0 (Microcal Inc., Northampton, MA, USA). Amorphous starch was prepared by heating a 10% starch solution at 95°C for 30min with continuous agitation and then drying at 100°C for 24h. The dried sample was ground into a free flowing powder using a RP 202 Pulaerit comminutator (Geoscience Instruments Corp., New York, NY, USA) with denatured alcohol as the solvent. The ground sample was air dried for 24h and passed through a 250μm sieve.

3.2.8.2 Determination of 'A' and 'B' polymorphic composition

The proportions of "A" and "B" polymorphs in the starches were estimated by the method outlined by Davydova *et al.* (1995). Different amounts (0-100%) of potato ['B' type] starch were thoroughly mixed with proportionate amounts (100-0%) of waxy corn ['A' type] starch (moisture contents were adjusted to 16%, w/w, a week prior to the experiment and left to equilibriate in sealed containers). The

diffractogram of each mixture was taken (with the same diffractometer settings as above) and the area under the peak 5.54 2θ angle was calculated using computer peak fitting software Jade – Version 2.1 (Materials Data Inc., Livermore, CA, USA) and Origin – Version 6.0 (Microcal Inc., Northampton, MA, USA). A standard series was created as the peak area ratio (area of the peak at 5.54/total peak area) vs. the amount of potato starch ('B' polymorph) in the mixture (**Fig. 1-D** in **Appendix 1**).

3.2.9 Swelling factor (SF)

The SF of the starches when heated to 50–95°C in excess water was measured according to the method of Tester and Morrison (1990). Starch samples (50mg, db) were weighed into screw cap tubes, 5mL of water was added, and heated in a shaking water bath at the appropriate temperature for 30min. The tubes were then cooled to 20°C; 0.5mL of blue dextran [M.W. 2,000,000] was added and mixed well. The tubes were then centrifuged at 1500g for 5min and the absorbance of the supernatant was measured at 620nm using a UV-visible spectrophotometer (LKB Novaspec – 4049 spectrophotometer (LKB Biochrom Ltd., Cambridge, England) against a reference without starch. This method measures only intragranular water and hence the true SF at a given temperature.

Calculation of SF was based on starch weight corrected to 10% moisture, assuming a density of 1.4mg/mL.

Free or interstitial plus supernatant water (FW) is given by

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

where A_r and A_s are absorbance of the reference and sample respectively.

The initial volume of starch (V_0) of weight W (in mg) is

$$V_0 = W/1,400$$

and the volume of absorbed intragranular water (V_1) is thus

$$V_1 = 5.0 - FW$$

hence the volume of the swollen starch granules (V_2) is

$$V_2 = V_0 + V_1$$

and $SF = V_2/V_0$

This can also be expressed by the single equation

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

The coefficient of variation of the method was generally less than 1%.

3.2.10 Extent of amylose leaching (AML)

Starches (20mg, db) in water were heated (50–95°C) in volume calibrated sealed tubes for 30min. The tubes were then cooled to ambient temperature (25-27°C) and centrifuged at 2000g for 10min. The supernatant liquid (1mL) was withdrawn and its amylose content was determined as described by Hoover and Ratnayake (2001). Percentage amylose leaching was expressed as mg of amylose leached per 100g of dry starch.

3.2.11 Pasting properties

A Brabender viscoamylograph (Model VA-V) equipped with a 700 cm cartridge (C.W. Brabender Instruments Inc., South Hackensack, NJ, USA) was used to study pasting properties at a concentration of 9% (w/v) and pH 5.5. The starch dispersions were stirred at 76rpm and heated at a rate of 1.5°C/min to 95°C, kept at this temperature for 30min, and then cooled to 50°C at a cooling rate of 1.5°C/min. The viscosity was expressed in terms of Brabender units (BU) and the pasting temperature was defined as the temperature at which the viscosity showed an increase in 10BU in the heating cycle. Duplicate measurements were used for this determination.

3.2.12 Differential scanning calorimetry (DSC)

Gelatinization parameters of native and retrograded starches were measured using a Seiko DSC 210 (Seiko Instruments Inc., Chiba, Japan) differential scanning calorimeter equipped with a thermal analysis data station.

3.2.12.1 Native starch

Water (11μL) was added with a microsyringe to starch (3.0 mg) in the DSC pans, which were then sealed, reweighed and allowed to stand for 2h at room temperature for moisture equilibrium. The scanning temperature range and the heating rates were 20-120°C and 10°C/min, respectively. In all measurements, the thermogram was recorded with an empty aluminum pan as the reference. The transition temperatures reported were the onset (T_o), peak (T_p) and

conclusion (T_c). The enthalpy (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 of starch (**Fig. 2-C** in **Appendix 2**).

3.2.12.2 Retrograded starch

Water (3μL) was added with a microsyringe to starch (3.0 mg) in the DSC pans, which were then sealed, reweighed and allowed to stand for 6h at room temperature for moisture equilibrium. Then the sealed pans were pre-scanned to gelatinize the starch. The scanning temperature and heating ranges were same as for the determination of gelatinization parameters. The heated pans were then cooled to room temperature and stored at: (1) 4°C for 24h, (2) 40°C for 24h, (3) 4°C for 24h and then at 40°C for 24h, (4) 4°C for 24h and then at 40°C for 24h and then at 4°C for 24h and then at 40°C for 24h. The pans were scanned under the conditions described for native starch.

3.2.13 Acid hydrolysis

Starches were hydrolyzed with 2.2*M* HCl at 35°C (1g starch/40mL acid) for 20 days. The starch slurries were shaken by hand daily to resuspend the deposited granules. At various time intervals, aliquots of the reaction mixture were neutralized and centrifuged (2000g). The extent of hydrolysis was determined by expressing the solubilized carbohydrates as a percentage of initial starch.

3.2.14 Enzymatic digestibility

Enzymatic digestibility (0-72h) studies on field pea starches were done using a crystalline suspension of porcine pancreatic α-amylase (Sigma Chemical Co., St. Louis, MO, USA) in 2.9*M* NaCl containing 3*mM* $CaCl_2$ (in which the concentration of α-amylase was 30 mg/ml and the specific activity was 790 units per milligram of protein (one unit was defined as the α-amylase activity which liberated 1mg maltose in 3min at 20°C, at pH 6.9). The procedure was essentially that of Knutson *et al.* (1982). Starch (100mg, db) was suspended in distilled water (25mL) and 5mL aliquots were placed in a constant temperature water bath (HAAKE SWB20, HAAKE Mess Technik GmbH u. Co., Karlsruhe, Germany) at 37°C. Then, 4.0mL of 0.1*M* phosphate buffer (pH 6.9) containing 0.006M NaCl were added to the slurry. The mixture was gently stirred before adding α-amylase suspension (12 units/mg of starch). The reaction mixtures were shaken by hand every 6h to resuspend the deposited granules. Then, 1mL aliquots were removed at specific time intervals (0, 24, 48 and 72h), pipetted into 0.2mL of 95% ethanol, and centrifuged (IEC-HN II Centrifuge, International Equipment Co., Needham, MA, USA) at 1000g. Aliquots of the supernatant were analyzed for soluble carbohydrate by the Dubios *et al.* (1956) method. Percentage hydrolysis was calculated as the amount (mg) of maltose released per 100mg of dry starch. Controls without enzyme but subjected to all the other experimental conditions were run simultaneously to eliminate the effects of spontaneous hydrolysis of starch (Hoover and Vasanthan, 1994).

3.2.15 Freeze-thaw stability

Aqueous suspensions of starches (6%, w/v) were rapidly heated to 95°C under constant agitation. These suspensions were then kept at 96°C for 30min before being cooled to 25°C. The gels thus obtained were subjected to cold storage at 4°C for 24h (to increase nucleation) and then frozen at –16°C (to increase propagation). The gels frozen at –16°C for 24h were thawed at 25°C for 6h and then refrozen at –16°C. Six freeze-thaw cycles were performed. The exuded water, at the end of each cycle, was gravimetrically determined by vortexing the thawed gels for 15 sec, followed by centrifugation at 1000g for 20 min.

3.2.16 Statistical Analysis

All determinations were replicated three times (unless otherwise indicated) and mean values and standard deviations are reported. Analyses of variance were performed and the mean separations were done by Tukey's HSD test at $p < 0.05$ using SigmaStat® (Version 2.0) statistical software (Jandel Scientific, Inc./SPSS, Inc., Chicago, IL, USA).

4 Results and discussion

4.1 Morphological characteristics of granules

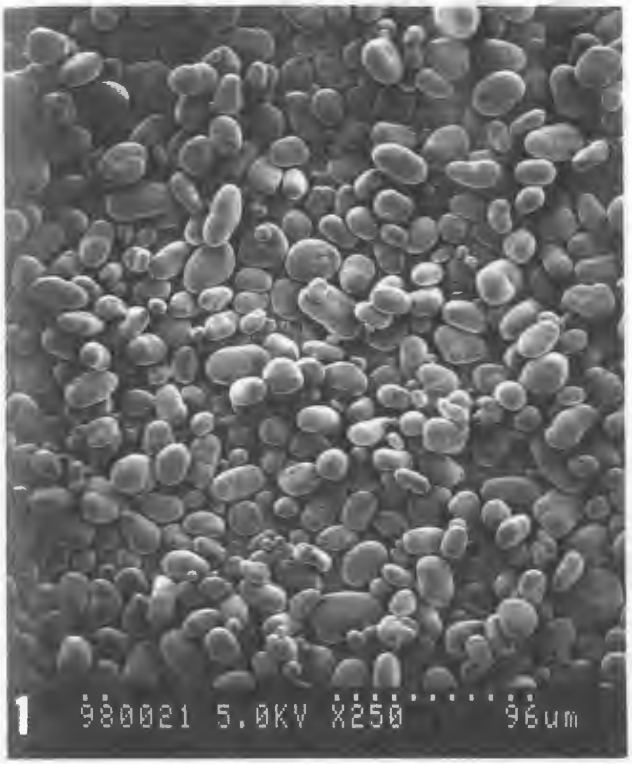
SEM examination of field pea starches showed that starches from all four cultivars had irregular shapes, which varied from round to oval to elliptical (**Fig. 4-1**). A variability of 5-25 μm was observed in the size (larger diameter) of the starch granule. This value was smaller than those reported for most of the other legume starches (12-65μm) (Hoover and Sosulski, 1991; Hoover *et al.*, 1997) and smooth peas (25-66μm) (Czuchajowska *et al.,* 1998), but comparable with lentil (2.5-25μm) (Hoover and Manuel, 1995). The surfaces appeared to be smooth and showed no evidence of fissures.

4.2 Chemical composition

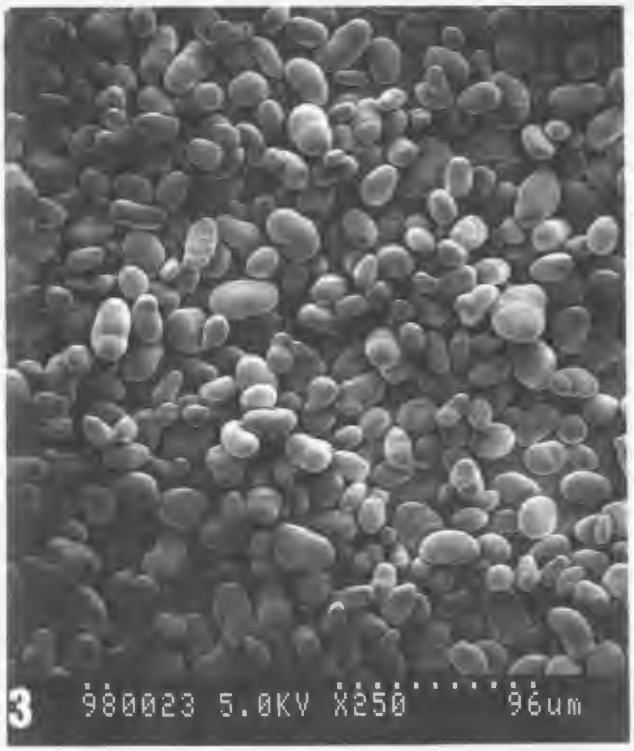
The data on composition and yield are presented in **Table 4-1**. The purity of the starches was judged based on composition and electron microscopic examination. The yield of starch (32.7-33.7%) was within the range (18-45%) (Hoover and Sosulski, 1991) reported for most other legume seeds.

The nitrogen content was in the range of 0.04-0.07%. This low value indicated the absence of non-starch lipids (lipids associated with endosperm proteins). Therefore, total lipids (0.28-0.34%) (**Table 4-1**) obtained by acid hydrolysis mainly represented the free and bound starch lipids (Vasanthan and Hoover, 1992b). The total lipid contents of the four starches were beyond the range reported for field pea (cv. Trapper) [0.09%] by Hoover *et al.* (1988) and most

Fig. 4-1 - Scanning electron micrographs of field pea starches: (1) Carneval, (2) Carrera, (3) Grande, (4) Keoma.


1
980021 5.0KV X250
96um


2
980024 5.0KV X250
96um


3
980023 5.0KV X250
96um

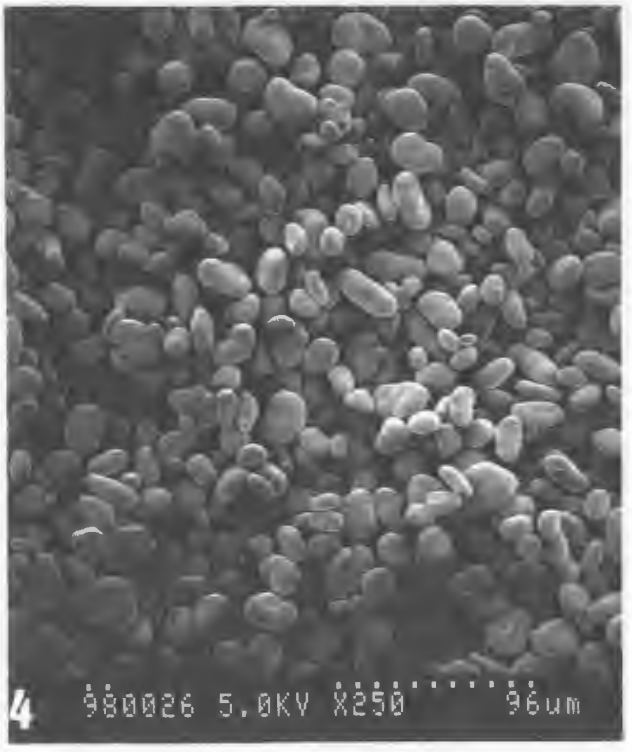

4
980026 5.0KV X250
96um

Table 4-1- Chemical composition (%) and some of the properties of field pea starches

Component/characteristic	Composition (%)[a] or property			
	Carneval	**Carrera**	**Grande**	**Keoma**
Yield (% initial material)	33.7 ± 1.5^{p}	33.2 ± 1.6^{p}	32.7 ± 1.5^{p}	33.5 ± 1.3^{p}
Moisture	13.3 ± 0.11	9.2 ± 0.08	12.3 ± 0.07	11.2 ± 0.09
Ash	0.03 ± 0.01^{p}	0.04 ± 0.01^{p}	0.03 ± 0.01^{p}	0.14 ± 0.01^{q}
Nitrogen	0.04 ± 0.01^{p}	$0.06 \pm 0.00^{p,q}$	0.04 ± 0.01^{p}	0.07 ± 0.01^{q}
Lipid				
Solvent extracted				
Chloroform-methanol[b]	0.05 ± 0.00^{p}	0.05 ± 0.00^{p}	0.05 ± 0.01^{p}	0.05 ± 0.00^{p}
n-Propanol-water[c]	0.27 ± 0.04^{p}	0.25 ± 0.04^{p}	0.24 ± 0.03^{p}	0.29 ± 0.03^{p}
Acid hydrolyzed[d]	0.31 ± 0.03^{p}	0.30 ± 0.04^{p}	0.28 ± 0.02^{p}	0.34 ± 0.04^{p}
Amylose content				
Apparent[f]	43.73 ± 0.03^{s}	43.49 ± 0.03^{r}	42.90 ± 0.03^{p}	43.17 ± 0.07^{q}
Total[e]	49.07 ± 0.14^{q}	49.57 ± 0.02^{r}	48.81 ± 0.06^{p}	$49.03 \pm 0.09^{p,q}$
Amylose complexed with native lipid[g]	10.88 ± 0.04^{p}	12.26 ± 0.06^{s}	12.11 ± 0.05^{r}	11.95 ± 0.07^{q}
Starch damage	1.73 ± 0.08^{p}	2.3 ± 0.04^{q}	2.50 ± 0.04^{r}	2.55 ± 0.08^{r}
Starch granule characteristics				
Granule shape	Oval to elliptical to round	Oval to elliptical to round	Oval to elliptical to round	Oval to elliptical to round
Granule size (μm)	5–25	5–25	5 - 25	5–25
Transmittance (%) at 640nm	20.6 ± 0.1^{q}	$20.7 \pm 0.0^{q,r}$	20.8 ± 0.0^{r}	20.2 ± 0.0^{p}

[a] All data reported on dry basis and the values followed by the same superscript in each row are not significantly different ($P < 0.05$) by Tukey's HSD test.
[b] Lipid obtained from native starch by chloroform-methanol 2:1 (v/v) at 25 °C (mainly unbound lipids).
[c] Lipid extracted by hot n-propanol-water 3:1 (v/v) from the residue left after chloroform-methanol extraction (mainly bound lipids).
[d] Lipids obtained by acid hydrolysis (24% HCl) of native starch (total lipids).
[e] Total amylose determined by I_2-binding after removal of free and bound lipids.
[f] Apparent amylose determined by iodine binding without removal of free and bound lipids.
[g] $\frac{\text{Total amylose - apparent amylose}}{\text{Total amylose}} \times 100$

other legume starches (Hoover and Sosulski, 1991) but was within the range reported for mung bean (0.32%) (Hoover *et al.*, 1997), and lentil (0.27-0.38%) (Hoover and Manuel, 1995) starches. The amounts of free and bound lipids were 0.05% and 0.24-0.29%, respectively (**Table 4-1**). The bound lipid contents of the field pea starches were beyond the range reported for beach pea (0.10%), grass pea (0.07%), green pea (0.12%) (Chavan *et al.*, 1999), pigeon pea (0.10%) (Hoover *et al.*, 1993) and lima bean (0.22%) (Hoover *et al.*, 1991) starches, but was close to that of mung bean (0.27%) (Hoover *et al.*, 1997) starch. The total amylose content of the field pea starches was in the range of 48.81-49.57% (**Table 4-1**). These values were much higher than those reported by Chavan *et al.* (1997) for beach pea (29.02%), green pea (36.70%), grass pea (36.37%) starches, and lower than those of smooth pea (52.6-57.0%) and wrinkled pea (94.0%) (Czuchajowska *et al.*, 1998), but was comparable to that of mung bean starch (45.3%) (Hoover *et al.*, 1997). Most of the data on the total lipid content of other legume starches have been obtained by the use of solvent systems that have proved inefficient in removing internal starch lipids. Therefore, a comparison of these results with published data of total starch lipids is not fully justified.

The apparent amylose content was in the range 42.90-43.73% (**Table 4-1**). A comparison of the apparent and total amylose contents (**Table 4-1**) showed that 10.88, 12.26, 12.11 and 11.95% of the total amylose content (which ranged from 48.81 to 49.57%) was complexed by native starch lipids in Carneval, Carrera,

Grande and Keoma, respectively. This was much higher than those reported for field pea (7.8%) (Hoover, 1998), beach pea (5.9%), grass pea (5.17%) (Chavan *et al.*, 1999), and pigeon pea (2.7%) (Hoover *et al.*, 1993) starches but was comparable to that of laird lentil (12.4%) (Hoover and Manuel, 1995), mung bean (12.1%) (Hoover *et al.*, 1997) and green pea (11.0%) (Chavan *et al.*, 1999).

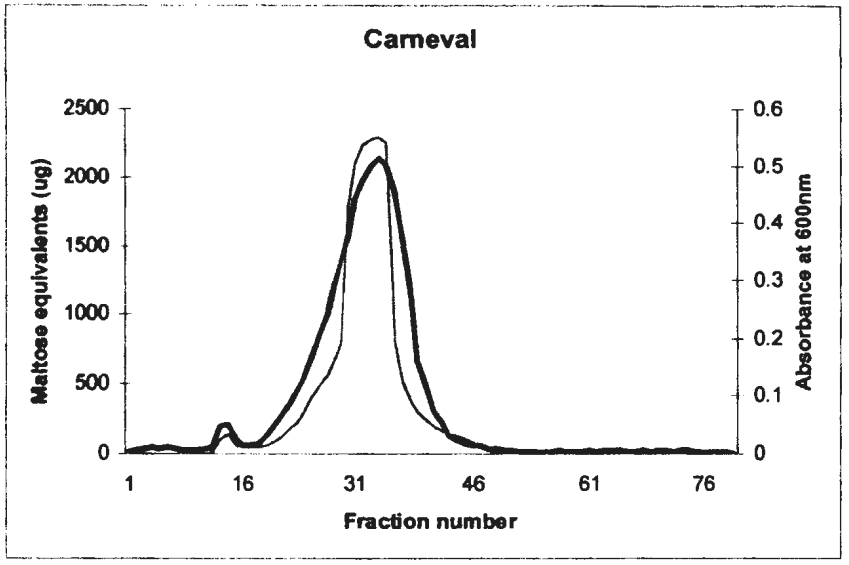
4.3 Starch structure

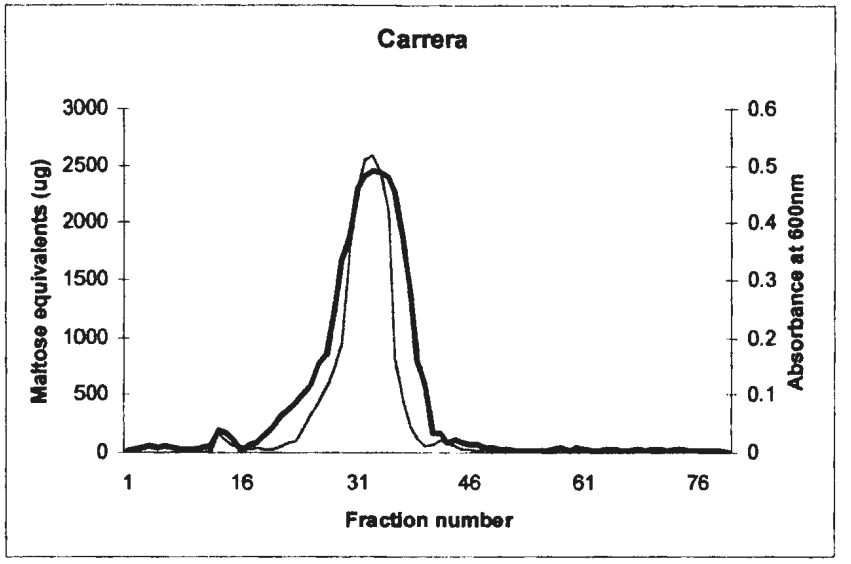
4.3.1 Molecular structure

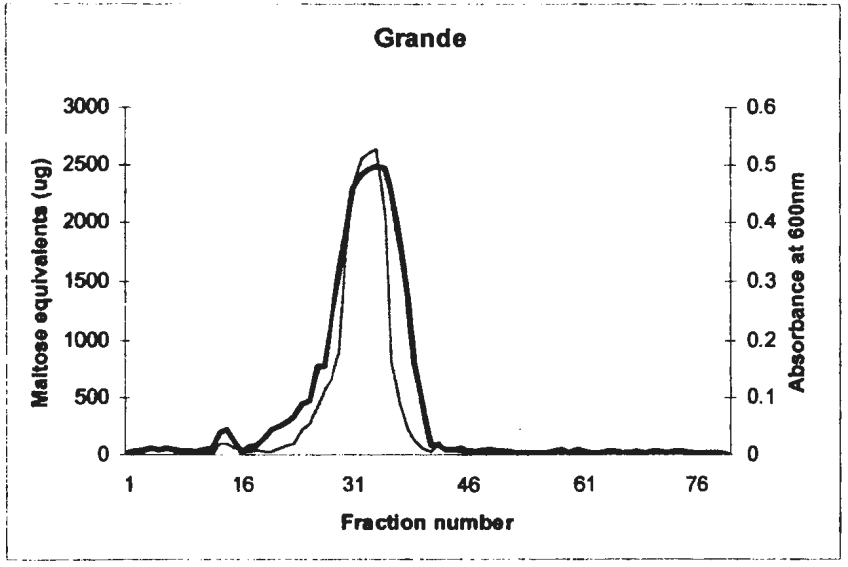
4.3.1.1 Degree of polymerization (DP) of amylose

The purity of isolated amylose from each cultivar was determined by GPC (**Fig. 4-2**). The area under the amylopectin peak (first peak) (**Fig. 4-3**) was negligible compared to the amylose peak (second peak) in all the chromatograms [the amylopectin peak was identified by running isolated field pea amylopectin through the GPC column with the same settings used for amylose and then analyzing the fractions for total carbohydrate (Dubios *et al.*,1956)]. This confirmed that the amylose isolated was not contaminated by amylopectin. All four starches exhibited similar trends with respect to both total carbohydrate and iodine affinity patterns the chromatograms. The degrees of polymerization of the isolated amyloses from the four cultivars of field pea are shown in **Table 4-2**. Carneval had the lowest DP of the four cultivars. The values of DP were

Fig. 4-2 - Gel permeation chromatographs of isolated field pea amyloses [Thick line (▬▬▬▬): iodine affinity and thin line (———): total carbohydrate].


Carneval
Maltose equivalents (ug)
Absorbance at 600nm
Fraction number
2500
2000
1500
1000
500
0
0.6
0.5
0.4
0.3
0.2
0.1
0
1
16
31
46
61
76


Carrera
Maltose equivalents (ug)
Absorbance at 600nm
Fraction number
3000
2500
2000
1500
1000
500
0
0.6
0.5
0.4
0.3
0.2
0.1
0
1
16
31
46
61
76


Grande
Maltose equivalents (ug)
Absorbance at 600nm
Fraction number
3000
2500
2000
1500
1000
500
0
0.6
0.5
0.4
0.3
0.2
0.1
0
1
16
31
46
61
76

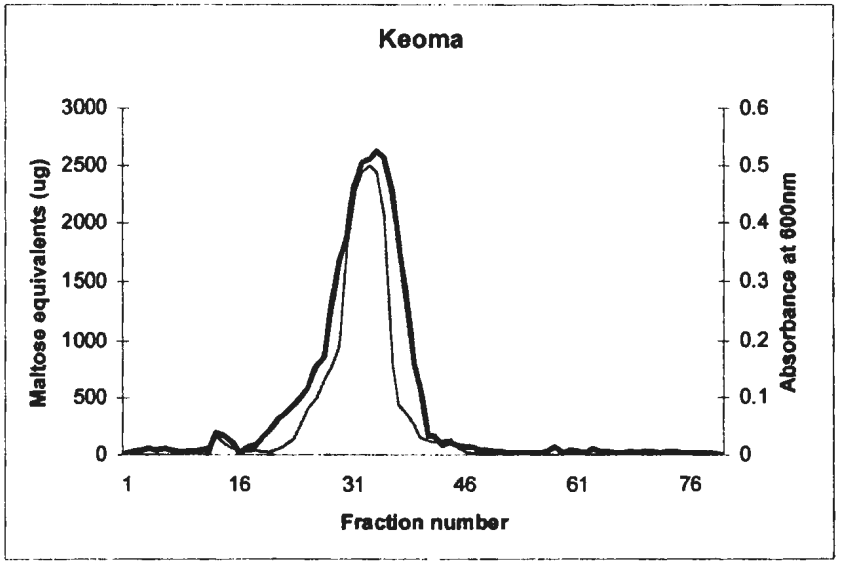

Keoma
Maltose equivalents (ug)
Absorbance at 600nm
Fraction number
3000
2500
2000
1500
1000
500
0
0.6
0.5
0.4
0.3
0.2
0.1
0
1
16
31
46
61
76

Fig. 4-3 - Gel permeation chromatograph of isolated field pea (Carneval) amylopectin (other three cultivars gave identical chromatograms).

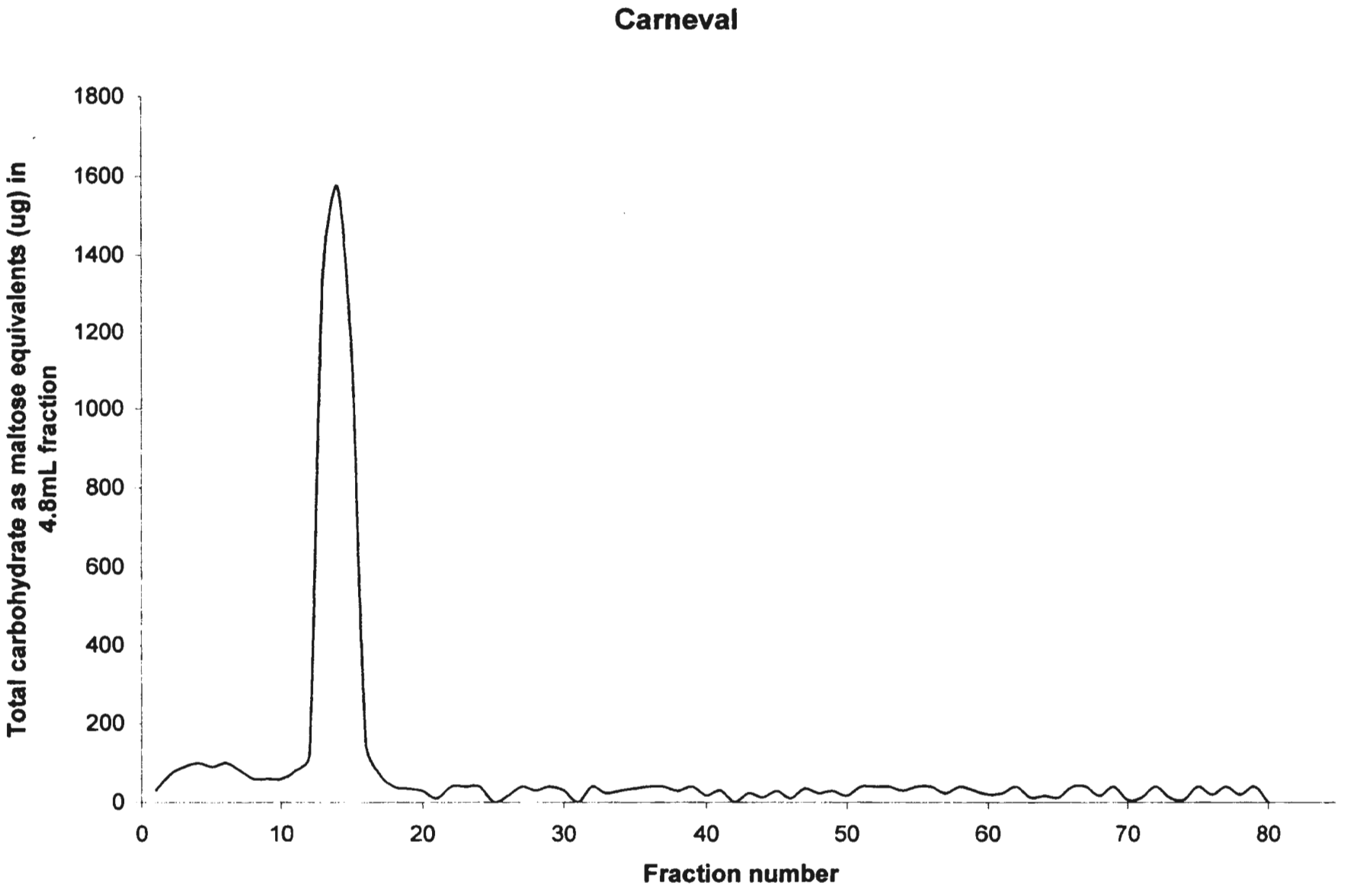

Carneval
Total carbohydrate as maltose equivalents (ug) in 4.8mL fraction
1800
1600
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
70
80
Fraction number

Table 4-2 - Degree of polymerization of field pea amylose

Cultivar	Degree of polymerization[a]
Carneval	1300 ± 11^{p}
Carrera	$1320 \pm 12^{p,q}$
Grande	$1322 \pm 15^{p,q}$
Keoma	1350 ± 10^{q}

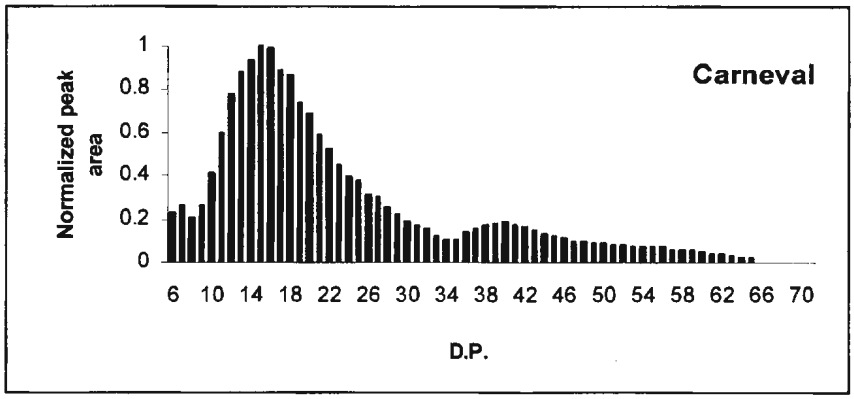
[a] The values followed by the same superscript are not significantly different by Tukey's HSD test at $P < 0.05$ level.

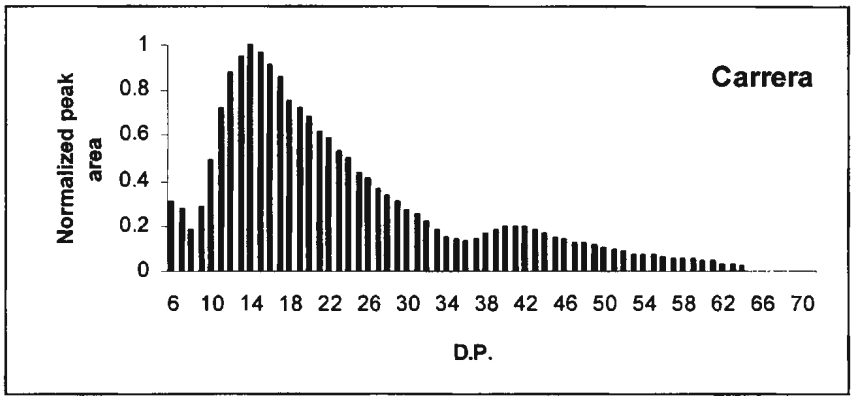
comparable to those reported for smooth pea starch (1300–1400) (Biliaderis *et al*, 1981).

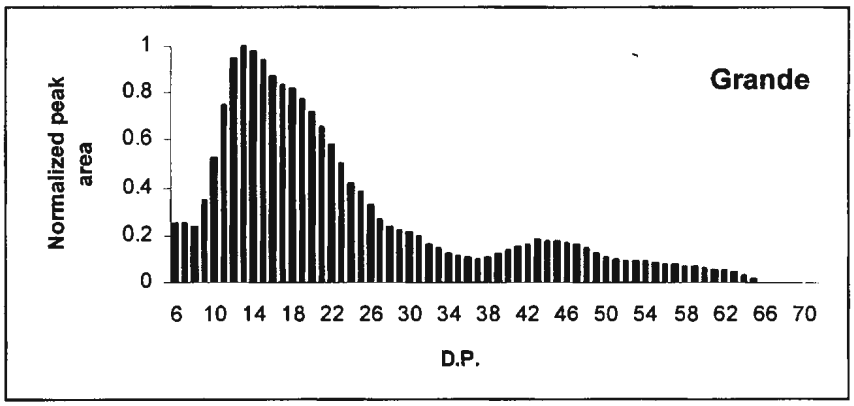
4.3.1.2 Chain length (CL) distribution of amylopectin

The normalized branch CL distributions of debranched amylopectins of the four starches are presented in **Fig. 4-4** and **Table 4-3**. The first peak in the bimodal peak distribution was at a peak CL of 14 for Carrera and Keoma, and at 13 and 15 for Grande and Carneval, respectively, while the second peak was at 43 for Grande and Keoma and 42 and 40 for Carrera and Carneval, respectively. Among the starches, Keoma had the largest average CL of DP 24.2. Grande, Carrera and Carneval had CL of DP 23.0, 23.1 and 22.9, respectively. Grande had a larger proportion (18.6%) of short branch chains (DP 6-12) in comparison to Carrera, Carneval and Keoma (17.2, 16.2, 17.0, respectively). Keoma had a larger portion (19.4%) of long branches (DP $\geq$ 37) than did Carrera (16.2), Carneval (16.4) and Grande (16.9%). The maximum detectable chain length was higher in Keoma (DP 71) than in the other three starches (DP 64-65) (**Table 4-3**). The largest average chain length, the highest amount of longer branches and the highest maximum detectable chain length might be the reasons for Keoma to have a relatively higher %crystallinity (compared to Carrera and Grande) and 'B' polymorph composition (**Table 4-4**) compared to the other cultivars. The average chain lengths of all four starches were comparable to those reported for other legume starches (CL 20-26) (Biliaderis *et al.,* 1981), but were lower than the range reported for tuber (CL 25.8-28.6) (McPherson and

Fig. 4-4 - Normalized peak area chromatograms of isoamylase debranched amylopectins of field pea starches by the use of HPAEC-ENZ-PAD


Carneval
Normalized peak area
1
0.8
0.6
0.4
0.2
0
6 10 14 18 22 26 30 34 38 42 46 50 54 58 62 66 70
D.P.


Carrera
Normalized peak area
1
0.8
0.6
0.4
0.2
0
6 10 14 18 22 26 30 34 38 42 46 50 54 58 62 66 70
D.P.


Grande
Normalized peak area
1
0.8
0.6
0.4
0.2
0
6 10 14 18 22 26 30 34 38 42 46 50 54 58 62 66 70
D.P.

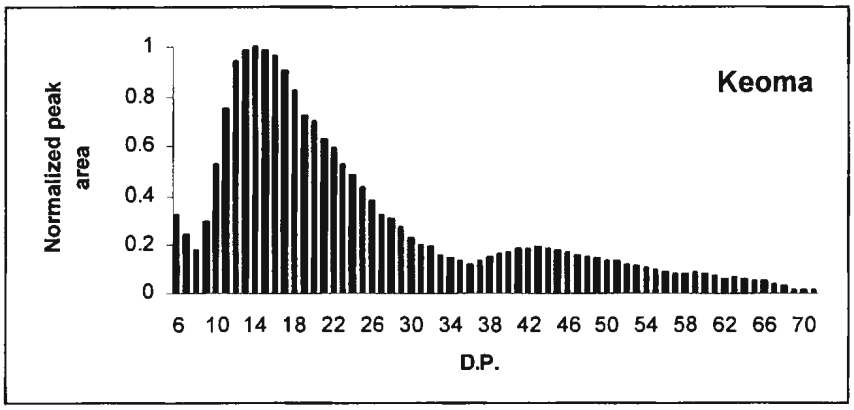

Keoma
Normalized peak area
1
0.8
0.6
0.4
0.2
0
6 10 14 18 22 26 30 34 38 42 46 50 54 58 62 66 70
D.P.

Table 4-3 - Branch chain length distributions of debranched field pea amylopectins

Starch source	First peak	Second peak	Distribution (%)*					
			DP 6-12	DP 13-24	DP 25-36	DP ≥ 37	Average chain length (CL)	Maximum detectable DP
Carneval	15	40	16.2 ± 1.9^{a}	52.9 ± 0.4^{c}	14.6 ± 1.7^{a}	16.4 ± 1.7^{a}	22.9 ± 0.5^{a}	65
Carrera	14	42	17.2 ± 0.3^{a}	48.2 ± 0.5^{a}	17.5 ± 0.8^{b}	16.2 ± 0.6^{a}	$23.1 \pm 0.6^{a,b}$	64
Grande	13	43	18.6 ± 1.4^{a}	$50.8 \pm 0.9^{b,c}$	13.9 ± 0.7^{a}	$16.9 \pm 0.1^{a,b}$	$23.0 \pm 0.3^{a,b}$	65
Keoma	14	43	17.0 ± 0.1^{a}	$48.5 \pm 1.6^{a,b}$	$15.1 \pm 0.5^{a,b}$	19.4 ± 1.1^{b}	$24.2 \pm 0.4^{a,b}$	71

* The total relative peak area was used to calculate percent distribution. Values followed by the same superscript in the same column are not significantly different ($P < 0.05$) by Tukey's HSD test.

Table 4-4 - Relative crystallinity and polymorphic composition of field pea starches[a]

Starch source	Relative crystallinity[b] (%)	'B' polymorphic composition[c] (%)
Carneval	25.06 ± 0.52^{s}	24.11 ± 0.31^{r}
Carrera	20.38 ± 0.47^{p}	22.11 ± 0.32^{p}
Grande	21.96 ± 0.45^{q}	23.32 ± 0.30^{q}
Keoma	$24.71 \pm 0.50^{r,s}$	25.56 ± 0.32^{s}

[a] The moisture content of the starches were 16% (w/w).

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

[c] Proportion of 'B' polymorph = Area of the peak at 5.54° (2θ)/total peak area.

Jane, 1999) starches. In general, it could be concluded that field pea amylopectin has high amounts of short (S) chains and low amounts of inner (B) chains in their structure.

4.3.2 Granular structure

4.3.2.1 Wide angle X-ray diffracton pattern

The field pea starches showed the characteristic 'C' type pattern of legume starches (Colonna *et al.*, 1982, Hoover and Sosulski, 1985, Gernat *et al.*, 1990, Davydova *et al.*, 1995). The X-ray pattern (**Fig. 4-5**) was characterized by strong intensity peaks at 5.9, 5.2, 5.0 and 3.8°A, and a weak intensity peak at 15.7°A ($2\theta = 5.54$).

4.3.2.2 Polymorphic composition

The peak at 15.7°A is characteristic of tuber starches. Gernat *et al.* (1990) have shown that the 'C' crystalline polymorph is a mixture of 'A' and 'B' unit cells, and legume starches contain pure 'A' and 'B' polymorphs in varying proportions. Both 'A' and 'B' polymorphs are composed of ordered arrays of parallel stranded double helices, which are formed by the short branches of amylopectin chains. These helices are closely packed in the 'A' type starches but loosely packed in the 'B' type starches. Furthermore, they also differ in intra-helical water content ('B' contains higher amounts than 'A') (Imberty, 1988; Imberty *et al.*, 1988). These ordered double helices form the crystalline lamella. Bogracheva *et al.* (1998) have concluded, by DSC and X-ray studies, that the 'A' and 'B'

Fig. 4-5 - X-ray diffraction patterns of native field pea starches [moisture content adjusted to 16%].

polymorphs of legume starches are present in the periphery and at the center of the granule, respectively. Hizukuri (1986) and Hizukuri *et al.* (1983) have shown that starches with amylopectins of short chain length (< 20 residues) exhibit 'A' type crystallinity, whereas those with amylopectins of longer average chain lengths show the 'B' pattern. The percentage 'B' polymorph in the starches followed the order: Keoma > Carneval > Grande > Carrera (**Table 4-4**). The values were lower than those reported (26-49%) by Davydova *et al.* (1995) and Cairns *et al.,* (1997) for smooth pea starches. The higher 'B' polymorphic content of Keoma could be attributed to its longer average amylopectin chain length (**Table 4-3**).

4.3.2.3 Relative crystallinity

The relative crystallinity of native field pea starches followed the order: Keoma ~ Carneval > Grande > Carrera (**Table 4-4**). The values were lower than those reported (Davydova *et al.*, 1995) for five varieties of smooth pea starches (26-32%). X-ray diffraction studies on maize starches of different amylose content (Cheetham and Tao, 1997) has shown that starch crystallinity is influenced by amylose content, average CL of amylopectin, and the mole percentage of short chain fractions of amylopectin (DP 10-13). The differences in total amylose content (**Table 4-1**), CL of amylopectin (**Table 4-3**), and the amount of short chains (DP 6-12) (**Table 4-3**) between the four starches were marginal and furthermore, differences in moisture content of the starches used for X-ray diffraction were also marginal. Therefore, the observed differences in crystallinity

probably represent differences in crystallite size and/or different orientations of the double helices within the crystallite.

4.4 Starch properties

4.4.1 Swelling factor (SF) and amylose leaching (AML)

The swelling factor (SF) and amylose leaching (AML) were investigated over the temperature range 50-95°C (**Table 4-5**). There were no significant differences in SF among the starches. The SFs (at 95°C) of the field pea starches were lower than those reported for beach pea (30.72), green pea (34.1) (Chavan *et al.*, 1999), mung bean (43.6), and CC gold lentil (31.0) but were comparable to that of laird lentil (26.0) (Hoover and Manuel, 1995) starch. Amylose-lipid complexes have been shown to influence granule swelling (Hoover and Manuel, 1996a; Maningat and Juliano, 1980; Tester and Morrison, 1990). The similar SF values for the four starches (**Table 4-5**) suggest that the level of lipid complexed amylose chains (Carrera > Grande > Keoma > Carneval) is not a factor influencing swelling in these starches. Tester and Morrison (1990) suggested that starch swelling is a property of amylopectin and amylose acts as a diluent. Since the starches exhibited significant differences in crystallinity (Keoma > Carneval > Grande > Carrera, **Table 4-4**), the granule swelling should have followed the order: Carrera > Grande > Carneval > Keoma. The results suggest that the differences in the level of crystallinity (**Table 4-5**) among these starches are too small to have any significant impact on SF.

Table 4-5 - Effect of temperature (°C) on the swelling factor (SF) and amylose leaching (AML) in field pea starches

Starch source	Temperature (°C)						
	50	60	70	80	85	90	95
Carneval							
SF	4.2 ± 0.21	8.5 ± 0.25	13.7 ± 0.16	19.4 ± 0.11	24.3 ± 0.04	26.5 ± 0.03	26.7 ± 0.21
AML	0.0 ± 0.00	10.5 ± 0.23^{q}	16.3 ± 0.17^{q,r}	19.6 ± 0.12^{q}	25.1 ± 0.03^{q}	26.3 ± 0.22^{q}	26.6 ± 0.16^{q,r}
Carrera							
SF	4.2 ± 0.22	8.6 ± 0.21	13.8 ± 0.23	19.4 ± 0.05	24.2 ± 0.05	26.4± 0.21	26.7 ± 0.24
AML	0.0 ± 0.00	10.1 ± 0.22^{p}	15.1 ± 0.12^{p}	18.1 ± 0.25^{p}	24.8 ± 0.24^{p}	25.1 ± 0.19^{p}	25.2 ± 0.10^{p}
Grande							
SF	4.1 ± 0.21	8.4 ± 0.22	13.8 ± 0.11	19.4 ± 0.10	24.2 ± 0.05	26.5 ± 0.16	26.7 ± 0.23
AML	0.0 ± 0.00	10.5 ± 0.23^{q}	16.0 ± 0.10^{q}	20.2 ± 0.08^{r}	25.7± 0.10^{r}	26.0± 0.12^{q}	26.2 ± 0.09^{q}
Keoma							
SF	4.1 ± 0.18	8.4 ± 0.22	13.3 ± 0.11	19.2 ± 0.20	24.1 ± 0.20	26.4 ± 0.20	26.5± 0.05
AML	0.0 ± 0.00	10.7 ± 0.15^{q}	16.6 ± 0.20^{r}	20.3 ± 0.06^{r}	25.5 ± 0.16^{r}	26.6 ± 0.20^{r}	26.8 ± 0.02^{r}

The values of AML followed by the same superscript, in the same column are not significantly different ($P < 0.05$) by Tukey's HSD test.

No significant differences ($P < 0.05$) were observed among the values for SF within the same column by Tukey's HSD test.

The extent of AML (at all the temperatures) of field pea starches followed the order: Keoma ~ Carneval ~ Grande > Carrera. AML (at 95°C) was much higher than reported (Chavan *et al.*, 1999) for beach pea (12.94), green pea (17.08), grass pea (19.07), but was lower than those reported for CC gold (35.5) and laird lentil (38.5) starches (Hoover and Manuel, 1995). Although Carrera had the highest amount of amylose, it had the lowest value for amylose leaching (AML). The main reason for this observation might be due to its high amount of lipid complexed amylose. Complexation of amylose with lipids reduces the leaching of amylose from the swollen granules. It is assumed that the amylose-lipid complex will retain its structure until the dissociation temperature of the complex is reached (Tester, 1997). The differences in AML in these starches are most probably influenced by the interplay between differences in amylose content, bound lipid content, and by the magnitude of the interaction between amylose chains within the native granule.

Both SF and AML increased dramatically between 60 and 85°C (**Table 4-5**). A similar trend has been observed within this temperature range for other legume starches (Schoch and Maywald, 1968; Tolmasquim *et al.,* 1971; Hoover and Sosulski, 1985; Hoover and Manuel, 1995; Chavan *et al.*, 1999). The rapid increase in SF and AML between 60 and 85°C (**Table 4-5**) may be due to an increase in molecular mobility of the amorphous region, which causes unravelling, and melting of the double helices present within the amorphous (double helices formed between amylose chains and between amylose and the

branched chains of amylopectin) and crystalline (double helices formed between the outer branches of amylopectin) domains.

4.4.2 Gelatinization parameters

The gelatinization transition temperatures [T_o (onset); T_p (midpoint); T_c (conclusion)] and the enthalpies of gelatinization (ΔH) of the four starches are presented in **Table 4-6**. T_o, T_p, T_c and $\Delta H/AP$ (enthalpy calculated on the basis of amylopectin content) did not vary substantially among the starches. However, the gelatinization temperature range (T_c–T_o) followed the order: Grande ~ Keoma > Carneval > Carrera. The results indicate that the numbers of double helices (in the amorphous and crystalline domains) that unravel and melt during gelatinization are nearly similar in all four starches. However, the differences in T_c-T_o suggest that the degree of heterogeneity of the starch crystallites within granules of Keoma and Grande is greater than those in Carneval and Carrera. The T_o, T_p, T_c and ΔH of the field pea starches were within the range reported for other legume starches (Hoover and Sosulski, 1991). Saunders *et al.* (1990) and Yuan *et al.* (1993) have postulated that longer chains in amylopectin could result in longer crystallites, and that more thermal energy is needed to break the kinetic barrier of longer crystallites due to increased crystalline order. However, this was not reflected in this study, in which Keoma had a greater proportion of long branches of amylopectin (DP>37) (**Table 4-3**) and had nearly equal transition temperatures and a ΔH value to the other starches. Zobel (1988a) reported that

Table 4-6 - Gelatinization[a] characteristics of native field pea starches

Starch source	Transition temperature[b] (°C)			$T_c - T_o$[c] (°C)	ΔH[d] J/g	$\Delta H/(AP)$[e] J/g
	T_o	T_p	T_c			
Carneval	61.4 ± 0.20^q	67.0 ± 0.22^p	76.0 ± 0.23^q	14.6 ± 0.11^q	11.5 ± 0.02^q	22.6 ± 0.12^q
Carrera	61.0 ± 0.22^p	66.8 ± 0.15^p	75.0 ± 0.11^p	14.0 ± 0.05^p	$11.4 \pm 0.08^{p,q}$	22.6 ± 0.10^q
Grande	61.0 ± 0.31^p	67.5 ± 0.16^q	76.0 ± 0.13^q	15.0 ± 0.08^r	11.2 ± 0.10^p	21.9 ± 0.14^p
Keoma	61.0 ± 0.15^p	67.0 ± 0.19^p	76.0 ± 0.17^q	15.0 ± 0.10^r	11.2 ± 0.08^p	22.0 ± 0.16^p

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

[b] T_o, T_p and T_c indicate the temperatures of the onset, midpoint and end of gelatinization respectively.

[c] $T_c - T_o$ indicates the gelatinization temperature range.

[d] Enthalpy of gelatinization.

[e] Enthalpy of gelatinization (ΔH) expressed on the basis of amylopectin content (AP). Values followed by the same superscript, in the same column are not significantly different ($P < 0.05$) by Tukey's HSD test.

there is no relationship between the amylose content and gelatinization temperature in 'C' type starches.

4.4.3 Pasting properties

The pasting properties of field pea starches are presented in **Table 4-7**. The four starches exhibited identical pasting temperatures, and exhibited only minor differences with respect to the viscosity at 95°C and to the increase in consistency during the holding period (at 95°C for 30min.). However, Carneval differed from the other starches with respect to: (1) the extent of the increase in viscosity on cooling to 50°C [85BU (Carneval) vs. 150-200BU (other cultivars)], and (2) the final viscosity at 50°C [230 BU (Carneval) vs. 300-350BU (other cultivars)]. The extent of granule swelling, the degree of friction among the swollen granules and the amount of leached amylose have been reported to contribute to starch pasting properties (Rasper, 1982). However, the polymeric nature of the molecules is known to be of primary importance in the determination of pasting properties of different starches (Shibanuma *et al.,* 1996). The gel-forming tendency of a starch paste is ascertained by its viscosity at 50°C (Miles *et al.*, 1985a). The field pea starches differed only marginally with respect to total amylose content (**Table 4-1**), granular swelling (**Table 4-5**) and amylose leaching (**Table 4-5**). Therefore, the above parameters appear not to be factors which determine the viscosity difference (at 50°C) and the extent of set-back between Carneval and the other three cultivars. Shibanuma *et al.* (1996)

Table 4-7 - Pasting characteristics of field pea starches[a]

Starch source	Pasting temperature[b] (°C)	Viscosity[c] at 95 °C (BU)[d]	Viscosity[c] after 30 min at 95 °C (BU)[d]	Viscosity[c] at 50 °C (BU)[d]
Carneval	79.5 ± 1.5	70.0 ± 2.5^{q}	145 ± 4.5^{q,r}	230 ± 5.5^{p}
Carrera	79.5 ± 2.0	60.0 ± 5.5^{p,q}	150 ± 5.0^{r}	300 ± 6.0^{q}
Grande	79.5 ± 2.5	55.0 ± 4.8^{p}	150 ± 4.5^{r}	350 ± 6.5^{r}
Keoma	79.0 ± 2.0	55.0 ± 4.5^{p}	130 ± 5.0^{p}	300 ± 5.8^{q}

[a] Starch concentration 9% (w/v) and pH 5.5.

[b] No significant differences ($P < 0.05$) were observed among the values by Tukey's HSD test.

[c] The values followed by the same superscript in the same column are not significantly different ($P < 0.05$) from each other by Tukey's HSD test.

[d] Brabender units.

reported that there was a very strong positive correlation between the amylose chain length and pasting viscosity. This suggests that the difference in the DP of amylose (Carneval < Carrera ~ Grande ~ Keoma) (**Table 4-2**) is probably the major factor influencing the difference in viscosity (at 50°C) and set-back. The DP of amylose would influence the extent of recrystallization during the cooling cycle in the viscoamylogram, as the number of stable crystallites would decrease with decreasing amylose chain length. Moreover, the smaller proportion of DP 6-12 branches in amylopectin of Carneval (**Table 4-3**) also could contribute to the observed differences in the gel forming characteristics during cooling cycle between Carneval and the other starches. Jane and Chen (1992) reported the long branch chain amylopectin would form stronger gels due to increased stable molecular interactions compared to their shorter counterparts.

Zobel (1984) reported that viscosity at 95°C reflects the ease of cooking of the starch, while the change in viscosity during holding at 95°C indicates paste stability during cooking under relatively low shear. This suggests that Grande and Keoma starches cooked easily compared to Carrera and Carneval, while Carneval and Keoma had the highest paste stabilities. Carneval showed the highest cooked paste stability during cooling from 95°C to 50°C.

The pasting curves of the field pea starches were typical of those of legume starches (Hoover and Sosulski, 1991).

4.4.4 Starch hydrolysis

4.4.4.1 Acid hydrolysis

All four starches exhibited a two-stage solubilization pattern when subjected to acid hydrolysis (**Table 4-8**). A relatively higher rate of hydrolysis was observed during the first 5 days, followed by a lower rate between 6 and 20 days. After the 5^{th} day of hydrolysis (0 to 5^{th} day duration corresponds to the degradation of the amorphous regions of the granule), there were no significant differences in the apparent rate of hydrolysis among the four starches. This observation is comparable to that previously reported for other legume starches (Chavan *et al.,* 1999; Hoover *et al.,* 1993; Hoover *et al.,* 1997; Hoover and Manuel, 1995; Biliaderis *et al.,* 1981). The results obtained indicate that there is no significant difference in the degree of helical packing and orientation within the amorphous domains of the four field pea starches. Morrison *et al.* (1993) have shown, by studies on lintnerized barley starches (covering a wide range of amylose and lipid contents), that lipid-complexed amylose chains are resistant to acid hydrolysis. On this basis, Carneval should have been hydrolyzed to a higher extent than the other field pea starches due to its lower bound lipid content (**Table 4-1**). However, the observed extent of hydrolysis, which was nearly equal in all four starches (**Table 4-8**), suggests that bound lipid content is not a factor influencing acid hydrolysis in these starches. The effect of very small amounts of bound lipids present in the field pea starches may be too insignificant to change the strong hydrolytic action of 2.2*N* HCl on the presumably larger amorphous regions

Table 4-8 - Acid hydrolysis of native field pea starches

Starch source	% hydrolysis at the end of each day										
	0	1	2	3	4	5	8	12	15	18	20
Carneval	0.0±0.0	4.8±0.1	10.3±0.1	13.5±0.1	16.8±0.4	20.0±0.2	26.1±0.2	31.7±0.2	34.6±0.2	36.8±0.2	37.8±0.3
Carrera	0.0±0.0	5.1±0.1	10.3±0.1	13.8±0.1	16.8±0.1	19.7±0.1	26.8±0.2	31.9±0.2	35.1±0.2	36.2±0.2	36.5±0.3
Grande	0.0±0.0	5.5±0.1	10.8±0.1	14.5±0.1	17.8±0.2	20.4±0.2	26.3±0.2	31.8±0.2	35.7±0.3	37.5±0.3	38.3±0.3
Keoma	0.0±0.0	5.1±0.1	10.8±0.1	14.6±0.2	17.9±0.2	20.0±0.2	26.4±0.2	31.8±0.2	34.4±0.3	37.0±0.3	38.8±0.3

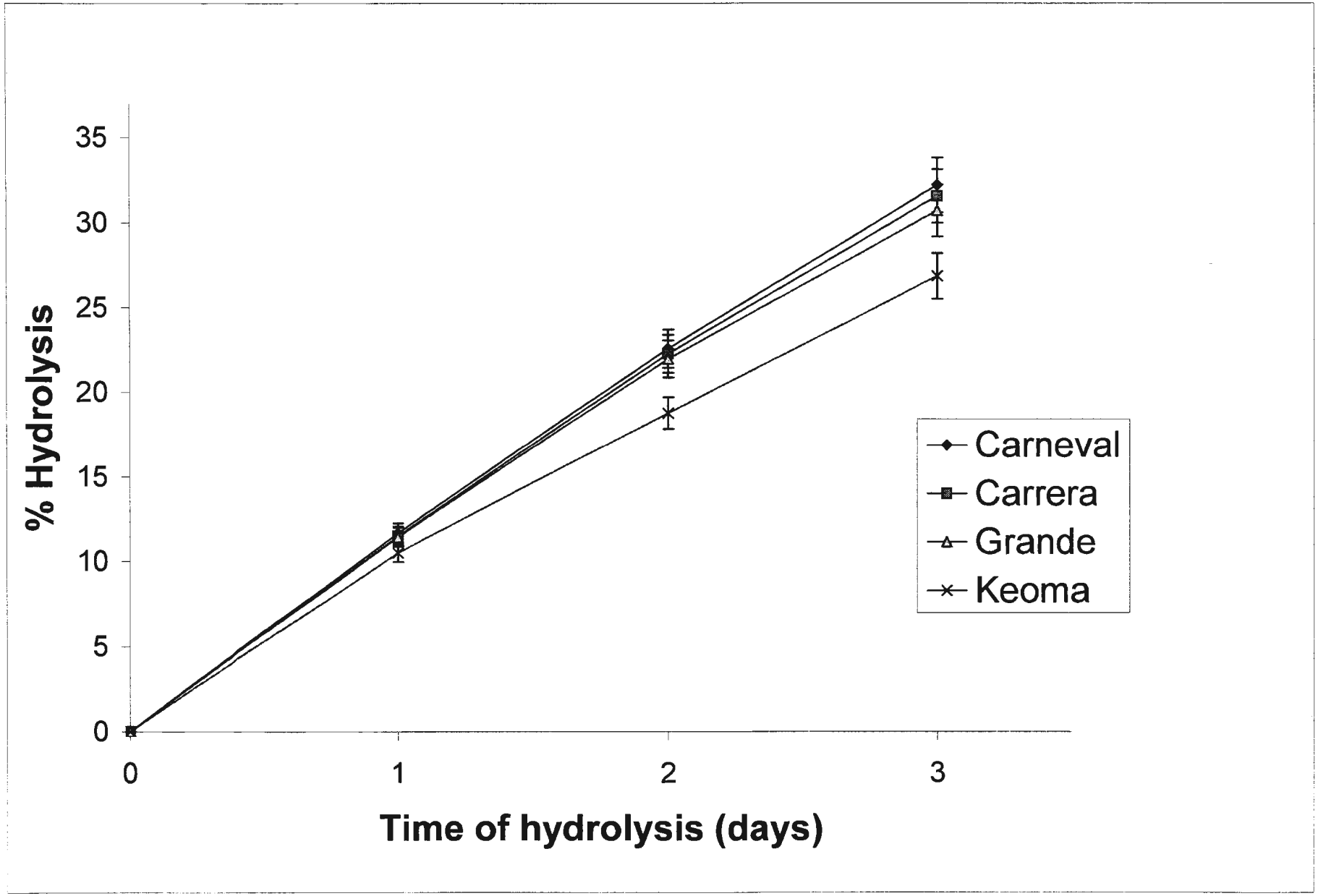
No significant differences ($p < 0.05$) were observed among the values in the same column by Tukey's HSD test.

of granules having higher amylose contents as only 10-12% of total amylose is complexed with native lipids. The results also suggest that the amount of double helices within the crystalline region and crystallite size are probably similar in all four starches. Thus, the differences that were observed in X-ray intensities (**Fig. 4-5**) probably reflect differences in crystallite orientation.

4.4.4.2 *In vitro* digestibility by porcine pancreatic α-amylase

The extent of hydrolysis of native field pea starches followed the order Carneval ~ Carrera ~ Grande > Keoma (**Fig. 4-6**). For instance, after 24h, Carneval, Carrera, Grande and Keoma were hydrolyzed to the extents of 22.2, 21.5, 20.7 and 18.2%, respectively. In comparison with the above starches, beach pea, grass pea, green pea, laird lentil, CC gold lentil and mung bean starches were hydrolyzed (in 24h) to the extents of 35, 22, 16, 14.5, 35 and 71%, respectively (Chavan *et al.*, 1999; Hoover and Manuel, 1995; Hoover *et al.*, 1997). Differences in the *in vitro* digestibility of native starches among and within species have been attributed to the interplay of many factors such as starch source (Ring *et al.,* 1988), granule size (Snow and O'Dea, 1981), amylose/amylopectin ratio (Hoover and Sosulski, 1985), extent of molecular association between starch components (Dreher *et al.,* 1984), degree of crystallinity (Hoover and Sosulski, 1985) and amylose lipid complexes (Hoover and Manuel, 1995; Holm *et al.*, 1983). Furthermore, it has been reported (Marsden and Gray, 1986; Franco *et al.,* 1988) that hydrolysis by α-amylase predominantly occurs in the amorphous regions of the granule. However, none

Fig. 4-6 - Time course of hydrolysis (%) of native field pea starches by porcine pancreatic α-amylase.


% Hydrolysis
35
30
25
20
15
10
5
0
0
1
2
3
Time of hydrolysis (days)
Carneval
Carrera
Grande
Keoma

of the above could explain the much lower rate and extent of hydrolysis (by α-amylase) of Keoma compared to the other cultivars. Planchot *et al.* (1997) have shown that there is a clear relationship between the hydrolysis rate of Lintnerized starches and their crystalline type. Regardless of morphology, particles with 'A' type crystallinity were found to be more susceptible to amylolysis than those with 'B' type. 'A' type lintners (waxy maize) showed the highest rates, whereas the rates for 'C' type lintners (mixtures of 'A' and 'B' type structures) were dependent on the 'A' type/'B' type ratio. Jane *et al.* (1997) have explained differences in the susceptibility between 'A' and 'B' type starches towards α-amylase in the following way: in 'A' type starches, the branch points are scattered in both amorphous and crystalline regions. Consequently, there are many short 'A' chains derived from branch linkages located inside the crystalline regions, which produces an inferior crystalline structure. This inferior crystalline structure containing α (1-6) linked branch points and the short double helices are more susceptible to enzyme hydrolysis, leading to "weak points" in the 'A' type starches. These weak points are readily attacked by α-amylase. However, in 'B' type starches more branch points are clustered in the amorphous region and furthermore, there are fewer short branch chains. Consequently, the crystalline structure is superior to that of 'A' type starches, and hence more resistant to α-amylolysis. Thus, the difference in the rate/extent of α-amylase hydrolysis (**Fig. 4-6**) between Keoma and the other starches could be attributed to the higher 'B' polymorph content in Keoma (**Table 4-4**), since the starches differ only marginally with respect to granule size, crystallinity, amylose content, % amylose

complexed lipids and extent of molecular association between starch components.

4.4.5 Retrogradation of starch gels

The extent of retrogradation during gel storage was monitored by determining changes in retrogradation enthalpy and changes in freeze-thaw stability.

4.4.5.1 Differential scanning calorimetry

The DSC parameters of the retrograded field pea starch gels are presented in **Table 4-9**. In all four starches, T_o, T_p, T_c and $\Delta H/AP$ of retrograded gels were lower than those for the gelatinization endotherms of their native counterparts (**Table 4-6**), and $T_c–T_o$ for retrogradation was broader than for the gelatinization endotherms (**Table 4-6**) in all the temperature treatments. The results showed that the extent of retrogradation of field pea starches is higher at 4°C compared to that at 40°C. This suggests that the process of initial nucleation is more important for the retrogradation of field pea starches compared to propagation, which takes place to a greater extent at 40°C. The melting temperature range ($T_c–T_o$) gives an indication of the quality and heterogeneity of the recrystallized amylopectin. Thus, a wide melting range would imply crystals with a large variation in stability, whereas a narrow range would suggest crystals of a more homogenous quality and similar stability. The lower $T_c–T_o$ at 40°C for 24h suggests that the crystallites are arranged more uniformly compared to that of at the lower temperature. The higher kinetic energy of the molecules at 40°C may

Table 4-9 - DSC studies on the retrogradation of field pea starch

Treatment[p] / parameter measured	Cultivar			
	Carneval	Carrera	Grande	Keoma
4°C for 24h				
ΔH_R	8.59 ± 0.05^{b}	8.61 ± 0.07^{b}	9.02 ± 0.06^{c}	8.21 ± 0.08^{a}
ΔH_R/AP	16.9 ± 0.04^{b}	17.1 ± 0.03^{c}	17.6 ± 0.05^{d}	16.1 ± 0.06^{a}
T_O	45.6 ± 0.12^{a}	45.8 ± 0.13^{a}	45.7 ± 0.27^{a}	45.7 ± 0.30^{a}
T_P	57.7 ± 0.40^{b}	57.0 ± 0.43^{a}	57.0 ± 0.00^{a}	$57.1 \pm 0.01^{a,b}$
T_C	78.7 ± 0.22^{a}	77.9 ± 0.07^{a}	77.1 ± 0.56^{a}	77.2 ± 0.40^{a}
$T_C - T_O$	33.1 ± 0.70^{c}	32.1 ± 0.06^{b}	31.4 ± 0.18^{a}	32.0 ± 0.12^{b}
40°C for 24h				
ΔH_R	6.21 ± 0.25^{c}	5.47 ± 0.32^{b}	5.17 ± 0.30^{b}	4.11 ± 0.25^{a}
ΔH_R/AP	12.2 ± 0.08^{d}	10.8 ± 0.21^{c}	10.1 ± 0.20^{b}	8.1 ± 0.15^{a}
T_O	60.2 ± 0.21^{a}	61.6 ± 0.14^{a}	60.1 ± 0.28^{a}	60.1 ± 0.25^{a}
T_P	69.5 ± 0.27^{a}	70.1 ± 0.31^{a}	70.1 ± 0.14^{a}	70.7 ± 0.25^{b}
T_C	78.3 ± 0.23^{a}	79.0 ± 0.21^{b}	78.4 ± 0.28^{a}	$78.6 \pm 0.24^{a,b}$
$T_C - T_O$	18.1 ± 0.21^{b}	17.4 ± 0.35^{a}	18.3 ± 0.24^{c}	18.5 ± 0.21^{d}
4°C for 24h, 40°C for 24h				
ΔH_R	$8.51 \pm 0.33^{a,b}$	9.14 ± 0.02^{c}	$8.96 \pm 0.28^{b,c}$	8.31 ± 0.08^{a}
ΔH_R/AP	16.7 ± 0.25^{b}	17.9 ± 0.00^{d}	17.5 ± 0.12^{c}	16.3 ± 0.04^{a}
T_O	57.35 ± 0.49^{a}	$58.45 \pm 0.07^{b,c}$	$58.6 \pm 0.42^{c,d}$	59.5 ± 0.07^{d}
T_P	66.2 ± 0.00^{a}	$67.0 \pm 0.14^{b,c}$	66.9 ± 0.21^{c}	67.7 ± 0.21^{b}
T_C	$76.9 \pm 0.00^{a,b}$	77.95 ± 0.21^{c}	76.6 ± 0.07^{a}	77.3 ± 0.08^{a}
$T_C - T_O$	19.6 ± 0.49^{b}	19.4 ± 0.04^{b}	18.0 ± 0.35^{a}	17.8 ± 0.14^{a}

[p] Samples of 3mg (db) of starch with 3µl of water, in hermetically sealed DSC pans were gelatinized and stored at different temperature combinations [1 - 4°C for 24h; 2 - 40°C for 24h; 3 - 4°C for 24h, 40°C for 24h; 4 - 4°C for 24h, 40°C for 24h, 4°C for 24h and 40°C for 24h]. Values carrying the same superscript, in the same row (within the same treatment) are not significantly different by Tukey's HSD test at $P < 0.05$.

ΔH_R = Retrogradation enthalpy (J/g of starch, db).

ΔH_R/AP = Retrogradation enthalpy (J/g) / %Amylopectin content.

T_O = Onset temperature (°C)

T_P = Peak temperature (°C)

T_C = Conclusion temperature (°C)

account for this observation. Temperature cycling (4°C for 24h and 40°C for 24h) lowers the extent of retrogradation and further decreases in T_c–T_o, suggesting a further increase in the uniformity of the crystallites formed.

The ΔH_R reflects the unravelling and melting of double helices formed during gel storage. Differences in ΔH_R among starches have been explained on the basis of amylopectin unit CL distribution (Fredriksson *et al.,* 1998; Kalichevsky *et al.*, 1990; Lu *et al.*, 1997; Shi and Seib, 1992; Ward *et al.,* 1994; Lai *et al.*, 2000). Liu and Thompson (1998) have shown by DSC studies that *du wx* (dull waxy) maize starch retrogrades faster than *wx* (waxy) maize starch. They have attributed this difference to the presence of a larger number of branch points in close proximity in the amylopectin clusters of *du wx* starch, which hinders large scale motion of the outer 'A' chains during retrogradation. This would favor formation, proper alignment and organization of double helices. At all of the temperature treatments, Keoma showed the lowest ΔH/AP compared to the other three starches. In this study, differences in amylopectin unit CL distribution among the starches were slight (**Fig. 4-4**). Since DSC monitors double helical dissociation (Cooke and Gidley, 1992), the energy required to dissociate double helices in retrograded Keoma would be much less (accounting for the low ΔH_R value) than for the others. Therefore, differences in ΔH_R between Keoma and the other three starches could only be due to differences in the number of branch points (Carneval ~ Carrera ~ Grande > Keoma) in close proximity in the disordered amylopectin clusters of these starches. The reduced number of branch points in

Keoma would impart greater flexibility to the outer 'A' chains, resulting in poorly formed and/or improperly aligned double helices during gel storage. Since DSC monitors double helical dissociation (Cooke and Gidley, 1992), the energy required to dissociate double helices in retrograded Keoma would be much less (accounting for the low ΔH_R value) than in the other starches.

4.4.5.2 Freeze-thaw stability

The freeze-thaw stability of a starch gel is evaluated by the amount (%) of water released (syneresis) when starch chains retrograde (reassociate) during the freeze-thaw cycles. Syneresis takes place due to both amylose and amylopectin retrogradation. The degree of syneresis of the starch gels is presented in **Table 4-10**. There were no significant differences in the extent of syneresis among these starches. In non-waxy starches, both amylose and amylopectin crystallization influence the degree of syneresis. Gidley and Bulpin (1987) showed that the kinetics of aggregation of amylose chains and the variation of gel strength with amylose concentration show a dependence on amylose chain length. These authors showed that precipitation and gelation occur for amylose CL of 250-660 residues, whereas for longer chains (>1100 residues) gelation predominates over precipitation. The results (**Table 4-10**) suggest that differences in amylose CL (**Table 4-2**), amylopectin unit CL distribution (**Table 4-3**), and the number of branch points (in close proximity to disordered clusters of amylopectin) among the starches were not large enough to cause significant differences in the extent of syneresis.

Table 4-10 - Freeze-thaw stability of field pea starches*

Starch source	Syneresis (%)					
	Number of freeze-thaw cycles					
	1	2	3	4	5	6
Carneval	18.6 ± 1.2	23.0 ± 1.4	24.0 ± 0.5	24.0 ± 1.4	25.5 ± 0.8	27.0 ± 2.1
Carrera	19.0 ± 1.5	25.0 ± 1.3	25.0 ± 0.7	26.0 ± 1.2	27.5 ± 1.8	28.0 ± 2.0
Grande	22.0 ± 1.1	22.0 ± 0.8	26.0 ± 1.9	26.0 ± 0.9	26.5 ± 1.9	28.0 ± 2.0
Keoma	22.0 ± 1.6	23.0 ± 2.0	26.5 ± 1.4	27.0 ± 2.0	28.0 ± 1.9	30.0 ± 2.1

* A 6% starch (db) solution.

No significant differences ($P<0.05$) were observed among the values in the same column by Tukey's HSD test.

Summary and conclusions

This study showed the relationships between structural and physicochemical properties of field pea starch.

The composition of non-carbohydrate components (lipids, ash and nitrogen) of field pea starch was comparable with values reported for other legume starches. However, the amylose content was higher than that previously reported for the other legume starches.

In the structural analysis of starch polymers, it was revealed that the degree of polymerization of amylose and the chain length distribution of amylopectin was within the range reported for most other legume starches. However, small differences among the cultivars were observed. The X-ray patterns were that of typical 'C' type starches. Relative percent crystallinity and polymorphic compositions were comparable to values reported for other legume starches.

Thermal behavior (SF, AML, gelatinization and pasting) of native field pea starches was comparable to those of most other legume starches with significant differences observed among the cultivars.

The susceptibility of field pea starches to acid and enzyme hydrolysis was comparable to values reported for other legume starches. The presence of a high amount of 'B' polymorph in Keoma makes it more resistant towards enzyme attack compared to the other three cultivars.

In general, the cultivar Keoma showed significantly different characteristics compared to the other three cultivars with respect to amylopectin branch chain length distribution, 'B' polymorphic composition, enzymatic digestibility, and amylopectin retrogradation. Furthermore, significant differences in pasting properties were observed between Carneval starch and starches from the other

cultivars. This suggests that, despite the fact that the plants were grown under same environmental and soil conditions, there might be differences in the starch properties among cultivars of the same species.

Retrogradation studies by DSC and freeze-thaw stability revealed that field pea starch was susceptible to a high rate and extent of retrogradation.

The experimental results of thermal and retrogradation behavior studies revealed that native unmodified field pea starch could not be used in food formulations due to their high retrogradation and unfavorable pasting properties.

Directions for future research

(1) Further studies on retrogradation of native, field pea starch with a wide range of storage times and temperatures would give a better understanding of the retrogradation behavior.

(2) Studies on the modification of starch by physical means would be important to obtain better thermal properties in the starch for field pea starch to be used in food products (specially as an economical source of commercial food starch). Annealing and/or heat moisture treatment could be used for the physical modification(s).

(3) Resistant starch (RS) and slowly digestible starch (SDS) have a considerable demand in low caloric food production. However, very few studies have been done on the possibility of the use of field pea starch in such products. Comprehensive investigations on the production of RS and/or SDS from field pea starch would be worthwhile because it would readily retrograde and become resistant to digestion *in vivo*.

Publications

R. Hoover and **W.S. Ratnayake** (2001) Determination of total amylose content of starch, In: Current protocols in food analytical chemistry, John Wiley and Sons, Inc., New York [*In press*].

W. Ratnayake, R. Hoover, F. Shahidi, C. Perera, and J. Jane (2000) Composition, molecular structure and physicochemical properties of starches from four field pea (*Pisum sativum* L.) cultivars. *Food Chemistry (In review).*

Honors and awards received for this research

George F. Stewart International Paper Competition, IFT Annual Conference (2000), Dallas, Texas – Won the third place for "Structure and physicochemical properties of field pea starches".

Student Monitorship, 2000 Institute of Food Technologists (IFT) annual conference and FoodExpo® (10-14 June), Dallas, USA.

Barrowman Biochemistry Travel Award (2000/2001), School of Graduate Studies, Memorial University of Newfoundland, St. John's, Canada.

Title of "Fellow of the School of Graduate Studies" in recognition of the continued academic excellence, Memorial University of Newfoundland, St. John's, Canada. 1999-2000.

Graduate Fellowship, Graduate School, Memorial University of Newfoundland, St. John's, Canada. (from 01st , Sept., 1998 to 31st Aug., 2000).

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Appendix 1

Fig. 1-A – Standard curve for amylose determination by Hoover and Ratnayake (2001) method.

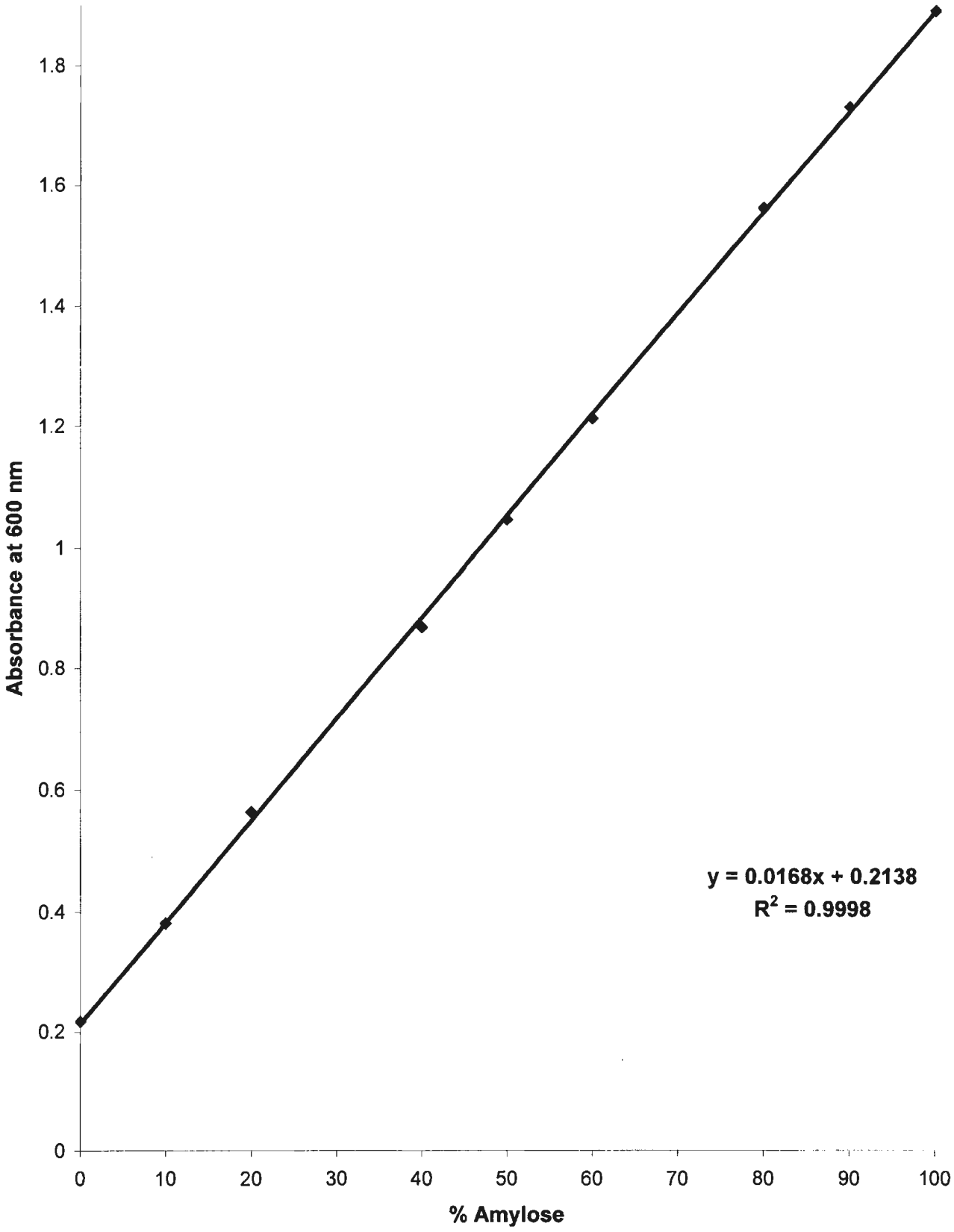

Absorbance at 600 nm
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
80
90
100
% Amylose
y = 0.0168x + 0.2138
R2 = 0.9998

Fig. 1-B – Standard curve for the determination of total reducing sugar by Bruner (1964) method.

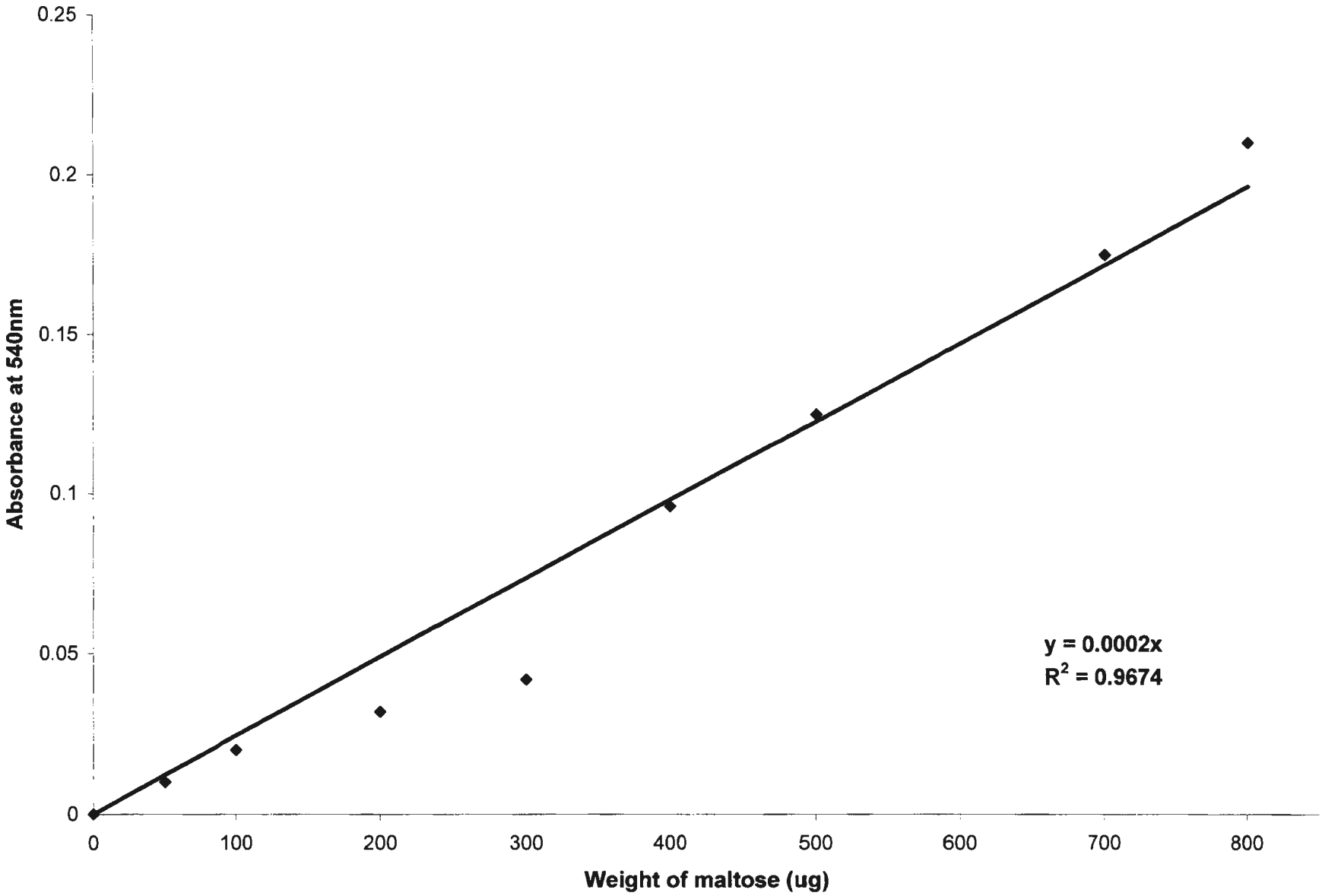

0.25
0.2
0.15
0.1
0.05
0
Absorbance at 540nm
0
100
200
300
400
500
600
700
800
Weight of maltose (ug)
y = 0.0002x
R2 = 0.9674

Fig. 1-C - Standard curve for the determination of total carbohydrate by Dubios *et al.* (1956) method.

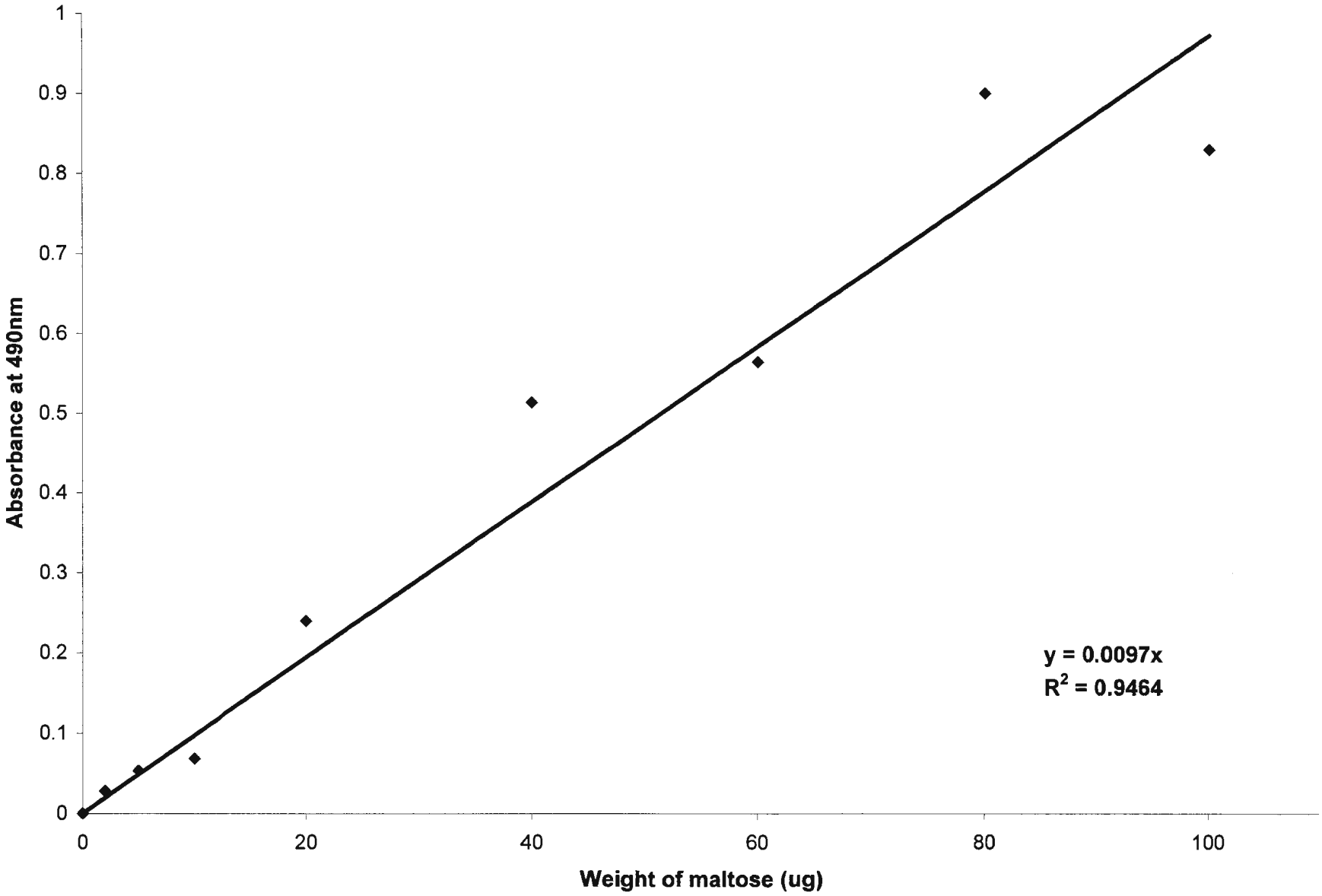
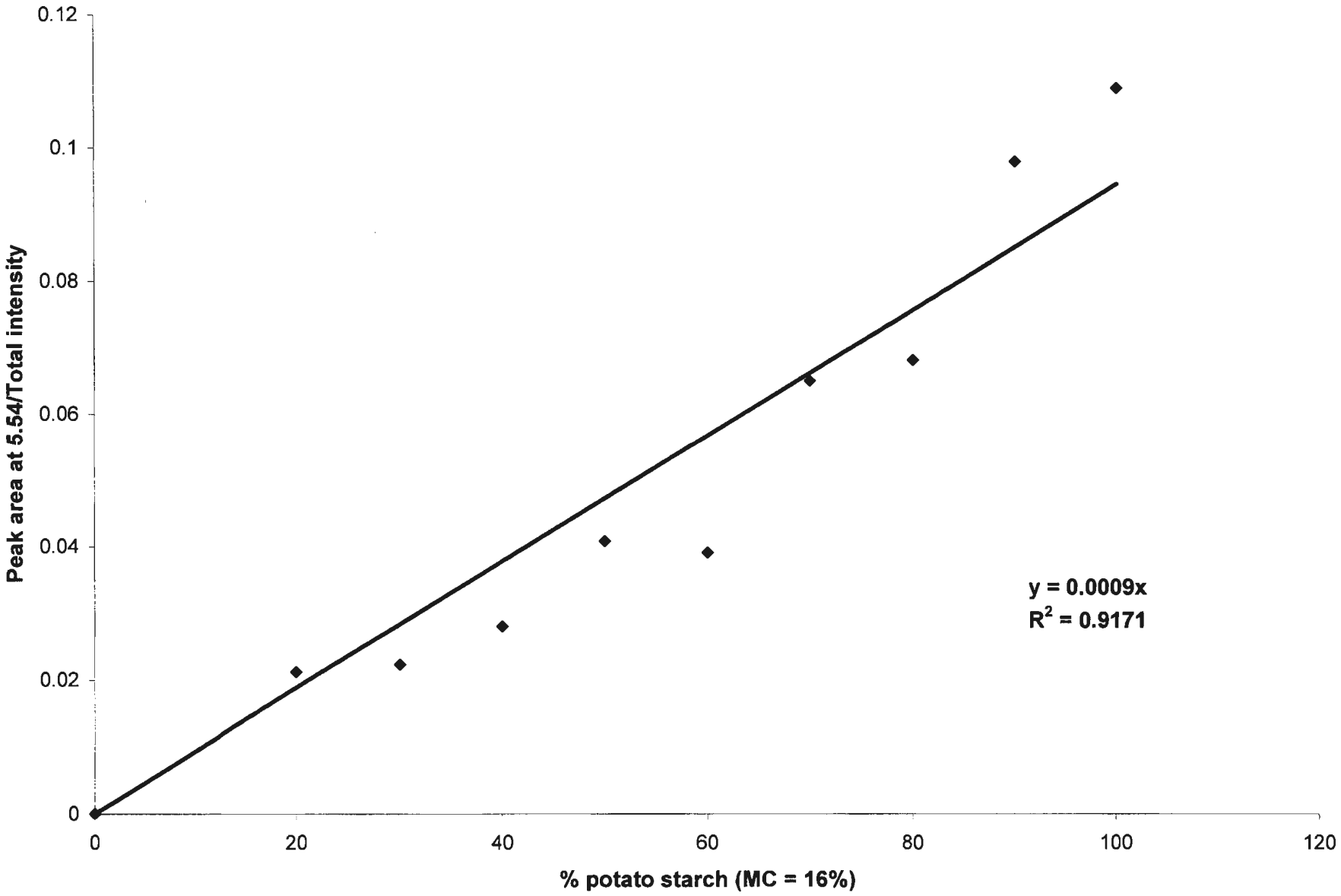

Absorbance at 490nm
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
Weight of maltose (ug)
y = 0.0097x
R2 = 0.9464

Fig. 1-D - Standard curve for the determination of 'B' polymorphic composition by Davydova *et al.* (1995) method [waxy maize starch was added in proportional amounts ranging from 100-0% to potato starch].


0.12
0.1
0.08
0.06
0.04
0.02
0
Peak area at 5.54/Total intensity
0
20
40
60
80
100
120
% potato starch (MC = 16%)
y = 0.0009x
R2 = 0.9171

Appendix 2

Fig. 2-A – An illustration of the equipment set-up for HPAEC-ENZ-PAD of debranched amylopectin.

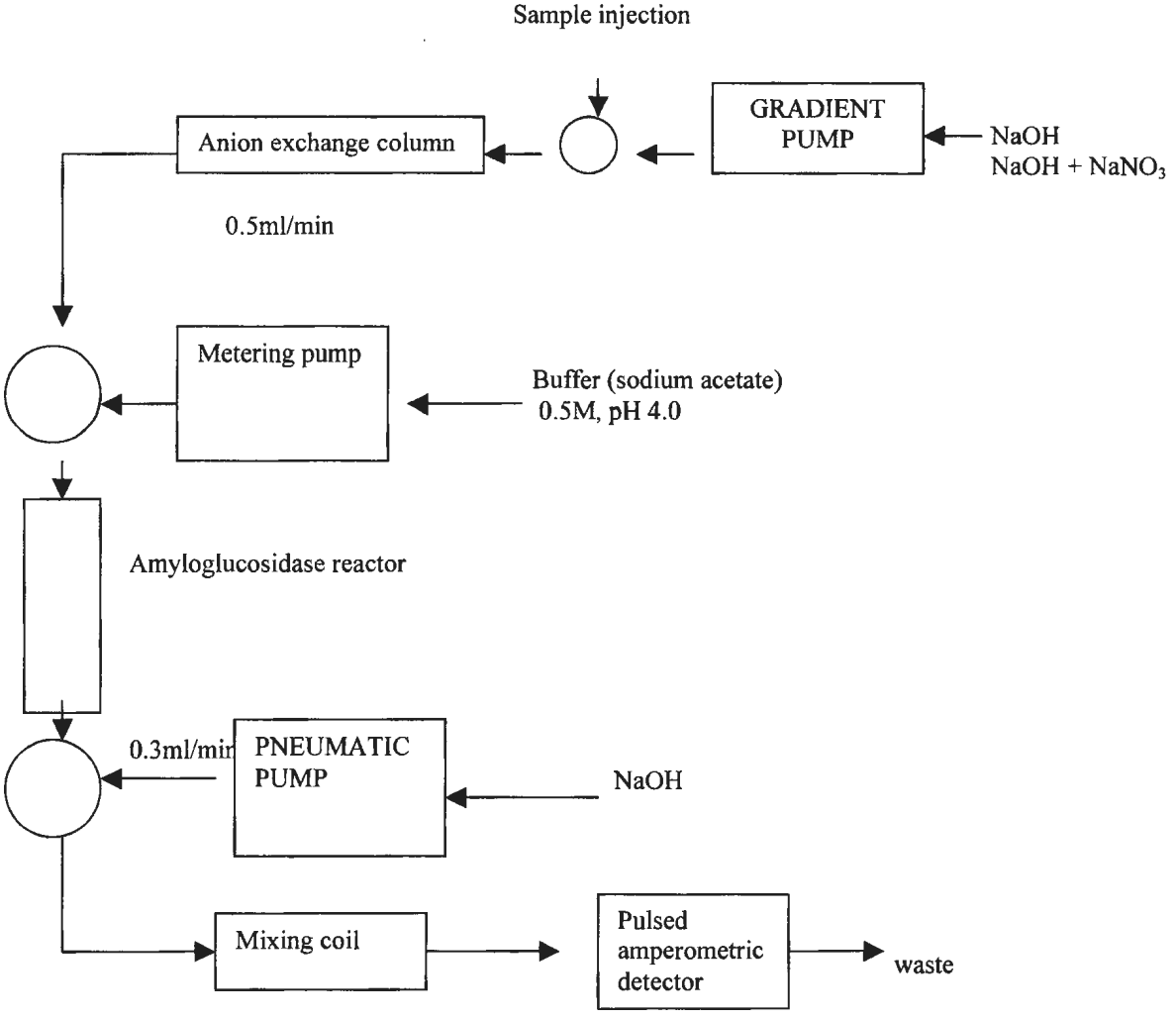
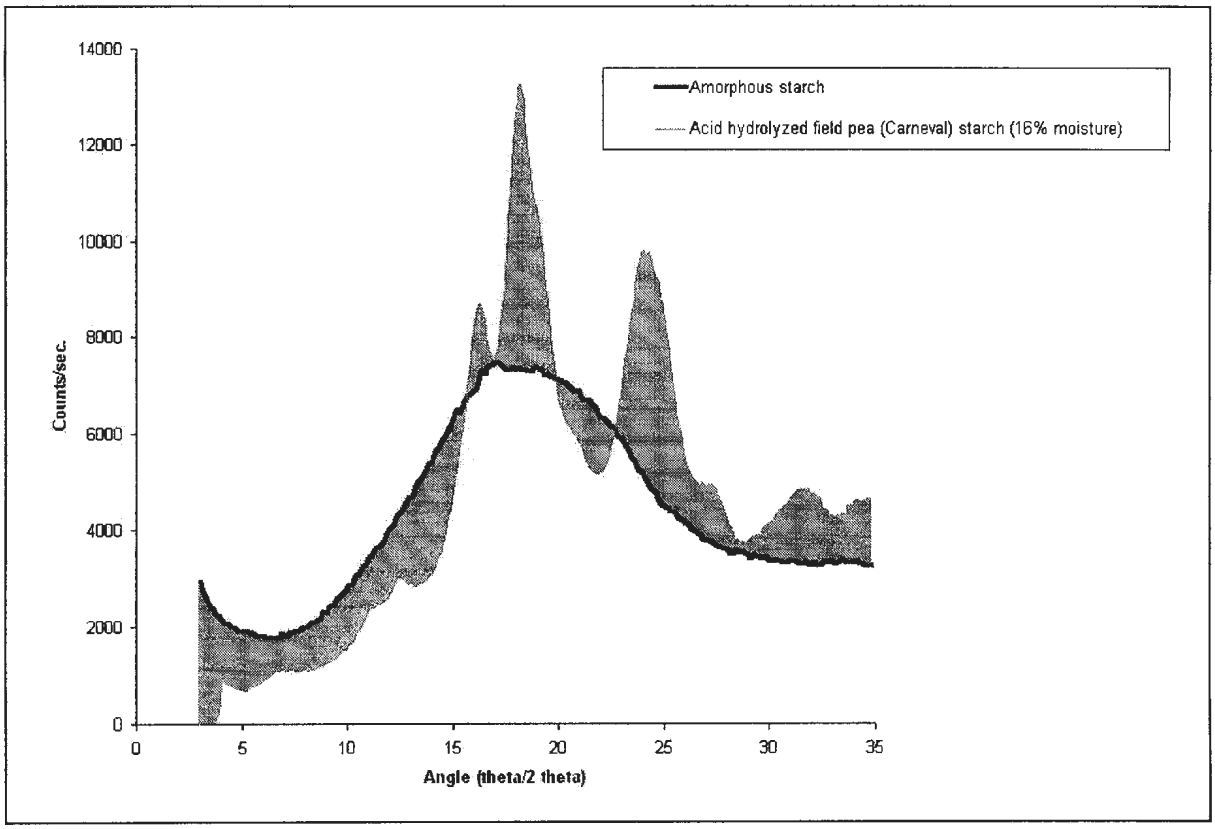

Sample injection
GRADIENT PUMP
NaOH
NaOH + NaNO3
Anion exchange column
0.5ml/min
Metering pump
Buffer (sodium acetate)
0.5M, pH 4.0
Amyloglucosidase reactor
0.3ml/min
PNEUMATIC PUMP
NaOH
Mixing coil
Pulsed amperometric detector
waste

Fig. 2-B - The method of calculation of %crystallinity by Nara *et al.*, (1978) procedure.

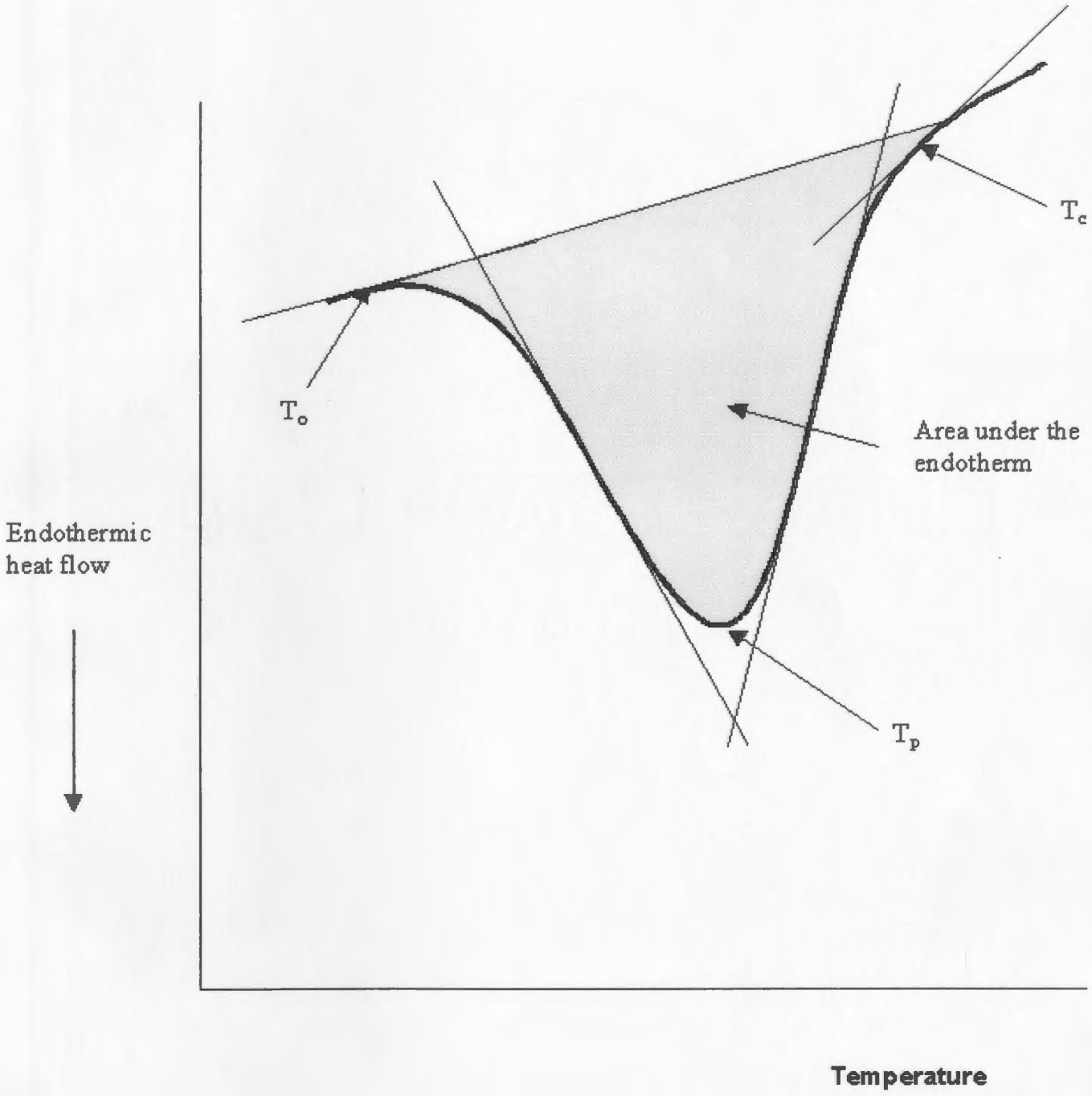


%Crystallinity = $\sum |I_s - I_a| / \sum |I_c - I_a| \times 100$, where $I_s - I_a$ = difference between the sample [I_s] and amorphous [I_a] intensities and $I_c - I_a$ = difference between the crystalline (quartz) [I_c] and amorphous [I_a] intensities.

The shaded area of the above figure represents $\sum |I_s - I_a|$, where I_s = intensity of acid hydrolyzed field pea starch (moisture content = 16%, w/w) and I_a = intensity of amorphous starch.

According to the same method, the value of $\sum |I_c - I_a|$ can be calculated [not shown in the figure].

Fig. 2-C – Calculation of gelatinization parameters in DSC [the area under the endotherm (shaded) is integrated to obtain the ΔH value]


T_c
T_o
Area under the endotherm
Endothermic heat flow
T_p
Temperature