HYDROLYSIS OF STARCH BELOW GELATINIZATION TEMPERATURE USING AN AMYLOLYTIC ENZYME

by

UTHUMPORN UTRA

Thesis submitted in fulfilment of the
requirements for the Degree of
Doctor of Philosophy

APRIL 2010

ACKNOWLEDGEMENTS

First of all I would like to take this opportunity to express my greatest appreciation to my supervisor, Prof. Abd. Karim Alias for his priceless guidance, understanding, moral support and invaluable advice throughout my research project. His great and inspirational supervision would not be unforgotten.

To all the laboratory staff members, thank you for the all the assistance during my research. I am also grateful to have such a courageous and high motivational working partner, Shariffa; thank you for everything. Not forgetting to my wonderful friends, Nadiha, Wahidah, Sufha, Ruri, Nizam, Mohd and the rest of the research lab for their advice and moral support.

I would like to acknowledge USM for rewarding me the USM fellowship. Last but not least, special thanks to my beloved parents for their endless love and support which always be the greatest encouragement in my life. Thank you for everything.

Uthumporn Utra

April 2010

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF SYMBOLS AND ABBREVIATIONS

Symbols	**Caption**
T_c	Conclusion temperature
T_g	Glass temperature
T_o	Onset temperature
T_p	Peak temperature
ΔH	Gelatinization enthalpy

Abbreviations	**Caption**
AM	Amylose
AMP	Amylopectin
DMSO	Dimethyl sulphoxide
DNS	Dinitrosalycylic
DE	Dextrose equivalent
DP	Degree of polymerization
DSC	Differential scanning calorimetry
GBSS	Granule bound-starch synthase
GSHU	Granular Starch Hydrolyzing Units
NaOH	Sodium Hydroxide
RCS	Refrigerated cooling system
RVA	Rapid Visco Analyzer
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
SSF	Solid state fermentation

LIST OF APPENDICES

HIDROLISIS KANJI DI BAWAH SUHU GELATINISASI MENGGUNAKAN ENZIM AMILOLITIK

ABSTRAK

Kebolehan enzim amilolitik (α-amylase dan glucoamylase) untuk menghidrolisis kanji pada keadaan asli (di bawah suhu gelatinisasi) dan kesannya ke atas organisasi molekul dan struktur kanji selepas dihidrolisis telah dikaji. Hidrolisis kanji asli di bawah suhu gelatinisasi telah dikaji bagi memahami tindakan enzim semasa hidrolisis. Kanji daripada sumber botani yang berbeza (jagung, kacang hijau, sagu dan kentang) telah dihidrolisis pada suhu 35°C selama 24 jam. Kemudian, kerintangan kanji bijirin dengan nisbah amilosa: amilopektin yang berbeza (jagung normal, jagung berlilin, jagung tinggi amilosa, beras dan beras berlilin) terhadap hidrolisis telah dinilai untuk melihat korelasi antara sifat fizikokimia dan struktur molecular kanji. Kanji gandum (granul kecil dan besar) telah dihidrolisis untuk mengkaji kesan saiz granul yang berbeza terhadap kadar hidrolisis. Pra-rawatan yang berbeza juga telah dilakukan terhadap kanji sebelum hidrolisis bagi memaksimumkan penukaran kanji kepada gula terfermen. Kesan rawatan haba (50°C selama 30 minit) terhadap tahap kerintangan kanji jagung, kacang hijau, sagu dan kentang untuk dihidrolisis telah dikaji. Kemudian, kesan penyingkiran lemak dan protin (pada permukaan/dalaman) kanji bijirin terhadap kadar hidrolisis juga telah dikaji. Lemak di dalam kanji telah dibuang (75% aqueous *n*-propanol selama 7 jam) dan protin pula telah dibuang dengan merawat kanji tersebut dengan NaOH (0.1%) dan protease (0.3% w/v). Kanji terhidrolisis telah dianalisa untuk menentukan kadar hidrolisis, sifat fizikokimia dan sifat berfungsi. Kanji jagung menghasilkan nilai dekstrosa yang paling tinggi (53%), diikuti kanji kacang hijau (36%), kanji sagu (15%) dan kanji kentang (12%). Analisis SEM menunjukkan kehadiran pori-pori

semulajadi pada permukaan kanji jagung dan kanji jagung terhidrolisis menunjukkan struktur granul berporos yang lebih banyak berbanding kanji lain. Untuk nisbah amilosa: amilopektin yang berbeza, jagung berlilin dan jagung normal didapati paling senang dihidrolisis iaitu ditukarkan kepada 54% dan 53% DE, diikuti kanji jagung tinggi amilosa (15%), beras berlilin (14%) dan beras (7%). Kanji gandum yang bergranul kecil juga menunjukkan DE yang lebih tinggi berbanding gandum bergranul besar dengan nilai DE masing-masing, 37% dan 27%. Kesemua kanji yang telah melalui proses pra-rawatan (rawatan haba, penyingkiran lemak, NaOH dan protease) menunjukkan peningkatan DE yang signifikan berbanding kanji asli selepas hidrolisis. Mikrograf SEM menunjukkan kanji yang telah dihidrolisis mempunyai granul berporos yang lebih banyak dan senang untuk dihidrolisis berbanding kanji asli. Taburan saiz partikel dan kandungan amilosa kanji terawat turun secara signifikan. Namun, tiada perubahan yang signifikan dapat dilihat pada corak X-ray kanji yang telah dihidrolisis, menunjukkan hidrolisis lebih cenderung untuk berlaku pada kawasan amorfus. Tindakan enzim menyebabkan perubahan yang signifikan terhadap kuasa pembengkakan dan keterlarutan, sifat pempesan dan gelatinisasi kanji. Pada asasnya, enzim ini berkebolehan untuk menghidrolisis kanji asli dan pra-rawatan sebelum hidrolisis telah meningkatkan kadar hidrolisis kanji.

HYDROLYSIS OF STARCH BELOW GELATINIZATION TEMPERATURE USING AN AMYLOLYTIC ENZYME

ABSTRACT

The action of amylolytic enzymes (α-amylase and glucoamylase) for their ability to hydrolyze starch in native granular state (below gelatinization temperature) and the effect of molecular organization and fine structure of starch polymers on the extent of hydrolysis had been studied. Hydrolysis of raw starches below gelatinization temperature was studied in order to understand the action of the enzyme during hydrolysis. Starches from different botanical origin (corn, mung bean, sago and potato) were chosen and hydrolysis was carried out at 35°C for 24 hours. Then, the susceptibility of cereal starches with different amylose: amylopectin ratios (normal corn, waxy corn, high amylose corn, rice and waxy rice) towards hydrolysis were evaluated in order to establish a correlation between physicochemical and molecular structure of starch. Wheat starch (large and small granules) was hydrolyzed as to evaluate the effect of granule size on the rate and extent of hydrolysis. Starches were also pre-treated with various pre-treatment before subjected to hydrolysis in order to give maximum conversion of starch to fermentable sugars. The effect of heat treatment (50°C for 30 minutes) on the susceptibility of corn, mung bean, sago and potato starches towards enzymatic hydrolysis was investigated. Then, the effects of removing lipid and protein (surface/indigenous) of cereal starches on the extent of hydrolysis were studied. Starches were defatted (75% aqueous *n*-propanol for 7 hours) and protein in starches was removed by treating the starches with NaOH (0.1%) and protease treatment (0.3% w/v). Hydrolyzed starches were analyzed for the degree of hydrolysis, physicochemical and functional properties. Corn starch yielded the highest value of

dextrose equivalent (DE) (53%), followed by mung bean starch (36%), sago starch (15%) and potato starch (12%). SEM micrograph showed the presence of natural pores on corn starch and hydrolyzed corn starch exhibited more porous granules compared to other starches. For different ratio of amylose: amylopectin, waxy corn and normal corn starch was the most susceptible being converted into 54% and 53% DE, followed by high amylose corn (15%), waxy rice (14%) and rice (7%). The small granule of wheat also showed higher DE compared to large wheat with DE at 37% and 27%, respectively. All the pre-treated starches (protease treatment, NaOH treatment, heat-treatment and defatting) showed significant increased in the DE compared to native starches after hydrolysis. SEM micrographs revealed that hydrolyzed treated starches had more porous granules and more susceptible to hydrolysis compared to native starches. The particle size distribution and amylose content of treated starches decreased significantly. However, no significant changes were observed in X-ray diffraction pattern of hydrolyzed starches, suggesting that hydrolysis preferentially occurred in amorphous region. The action of enzymes caused significant changes in swelling power and solubility, pasting and gelatinization properties. Evidently, the enzymes were able to hydrolyze granular starches and pre-treatment before hydrolysis significantly increase the rate of hydrolysis.

CHAPTER 1

INTRODUCTION

1.1 Background and rationale

Starch constitutes a major component of foods and also a raw material for used in the production of industrial products. Starch in its native form exists in relatively inert granular structures, which are composed of macromolecules arranged in polycrystalline state. Due to its semi-crystalline granular structure, starch is easily isolated through wet milling and it can be modified with various chemical or physical treatments (Whistler et al., 1984) in order to tailor it to the desirable functional properties suited to the applications. However, starches of different botanical origins have different characteristics, shapes, sizes and morphology (Oates, 1997) that will affect the functional properties and the resistance of the granules towards hydrolysis. Starch is the lowest priced and most abundant worldwide commodity. It is produced in most countries and is available at low cost in all countries. Its level price over many years is impressive and makes it especially attractive as an industrial raw material. Its production by wet milling industry has continued to increase and may increase at a faster rate as starch takes more of the sweetener market and as government subsidize ethanol production (Whistler et al., 1984).

The most common renewable fuel today is ethanol derived from corn grain (starch) and sugar cane (sucrose). Starch processing is a fairly mature technology utilizing enzymatic liquefaction and saccharification, which produces a relatively clean glucose stream that is then fermented to ethanol by *Saccharomyces* yeast. Liquefaction requires the starch to be treated at high temperature and elevated pressure to achieve complete gelatinization and granule disruption (Gray et al.,

2006). This is an energy intensive process requiring the addition of heat energy to starch slurries until the gelatinization temperature of the starch is exceeded. It has been reported that the liquefaction step contributed approximately 50% of the total energy consumption for the whole starch conversion processes. Industrial fermentation processes could be operated more cost-effectively by carrying out the direct hydrolysis of native starch without prior gelatinization (Oates, 1997).

Alternative to petroleum-derived fuels are being sought in order to reduce the world's dependence on non-renewable resources such as petroleum. In this regard, starch can become the alternative renewable source of energy. However, the current process to produce bio-ethanol (via conventional starch conversion process) is not economically attractive. This is due to energy-intensive process as mentioned earlier. The key to the establishment of a commercial process is a reduction in capital and operating cost of each of the unit operation. Therefore, a new process has to be developed where the energy consumption to convert starch to high value syrup (sugar) and energy source can be substantially reduced. In the view of energy costs, effective utilization of natural resources and direct hydrolysis of starch below gelatinization temperature is desirable.

In recent years, the importance of enzymic liquefaction of raw starch without heating has become well recognized, mainly from the viewpoints of energy saving and effective utilization of the biomass thereby reducing the cost of starch processing (Aggarwal and Dollimore, 1998). This has generated a worldwide interest in the discovery of several raw starches digesting amylase that does not require the gelatinization and can directly hydrolyze raw starch in a single step. The development of enzymes that function on raw and uncooked starch would improved overall process economics (Gray et al., 2006).

Typically, hydrolysis of granular starch by enzymes was often slowly, resulting in low degree of conversion of starch to fermentable sugars (Oates, 1997). According to Sarikaya et al. (2000), hydrolysis of native starch granules by α- and β-amylase is not very effective because starch granules are very resistant to amylolytic degradation and a long hydrolysis period is require for degrading the starch. Recently, a novel amylolytic enzyme (STARGEN 001) obtained from Genencor International (B.V., The Netherlands) is able to hydrolyze native granular starch below the gelatinization temperature. STARGEN 001 is an enzyme blend consisting of α-amylase from *Aspergillus kawachi* and glucoamylase from *Aspergillus niger*. The exo-activity of glucoamylase can drill sharp and deep pinholes into the granular starch while the endo-activity of α-amylase can widen the pinholes and degrade amylose at the same time. This perfect combination will catalyze the release of fermentable glucose continuously from granular starch and will concomitantly develop a starch hydrolyzing process without the need for starch gelatinization or liquefaction. This new technology has the potential to eliminate the need for high-energy processing of starch and provide more cost-effective for conversion of starch to other bioproducts and biomaterials such as glucose, high fructose corn syrup and ethanol. Benefits of using STARGEN 001 in the process would produce higher yield of oligomers (oligosaccharides, maltodextrin, maltose, glucose, etc.), energy saving, fewer steps of processing, require fewer chemicals, process simplification and saving on capital cost.

1.2 OBJECTIVES

General Objective

To study the action of an amylolytic enzyme for its ability to hydrolyze starch in its native (raw) granular state (below gelatinization temperature) and the effect of molecular organization in starch granule as well as fine structure of starch polymers on the extent of conversion to various oligosaccharides.

Specific Objectives

1. To conduct a systematic evaluation of the capability of an amylolytic enzyme to hydrolyze raw starch (at 35°C) from various botanical sources with at least one type of starch representing cereals, tubers, roots, legumes/seeds and palm starch.
2. To establish correlation between the amylose/amylopectin ratio, type/degree of crystallinity and fine molecular structure of amylose/amylopectin with degree of hydrolysis.
3. To find ways (via mild physical pre-treatment) to enhance degree of starch conversion (hydrolysis) to oligosaccharides using an amylolytic enzyme while maintaining the operation below gelatinization temperature (35°C-60°C).

1.3 Thesis Outline

Hydrolysis of raw starch below gelatinization temperature (35°C) using an amylolytic enzyme and pre-treatment of starch to enhance the degree of hydrolysis below gelatinization temperature is presented in this thesis. This dissertation is divided into nine chapters consist of general introduction and background, literature reviews, material and methods, results and discussion, general and overall conclusion as well as recommendation for future study.

CHAPTER 1 consists of general introduction and background of this project in which the current situations and challenges encountered by food industry regarding hydrolysis of starch and production of fermentable sugars/ethanol. The objectives and the rationale of this study are briefly discussed.

CHAPTER 2 focuses on the general literature review of starch, enzymes, hydrolysis and modification of starch. In the first part, the components, structure, functional properties and modification of starch are reviewed. Then, enzymes are divided into amylase, glucoamylase and STARGEN enzyme. Lastly, hydrolysis of starch in raw granular state is discussed. This research deals with hydrolysis of starch below gelatinization temperature in order to understand the action of amylolytic enzyme (STARGEN 001) on raw starches. Furthermore, starches were also pre-treated with various pre-treatment before subjected to hydrolysis in order to increase the degree of hydrolysis.

CHAPTER 3 consists of fundamental study of hydrolyzing starch from different botanical sources using STARGEN 001. Different starches from different botanical origin (corn, mung bean, sago and potato) would show different susceptibility to enzymatic attack below gelatinization temperature. The enzyme,

capable of hydrolyzing native granular starch, has been developed by proprietary technology and not much is known about this enzyme. Therefore, it is important to understand the action of this enzyme in hydrolyzing raw starch. Then, characterization and physicochemical properties of hydrolyzed starches were studied.

CHAPTER 4 focuses on hydrolyzing starches with different ratio of amylose: amylopectin (corn, waxy corn, high amylose corn, rice and waxy rice). The ratio of amylose: amylopectin is known to be one of the important factors in determining the susceptibility of starches to enzyme attack. Thus, the knowledge obtained would be used to correlate the susceptibility of starches to enzyme attack (below gelatinization temperature) with molecular structure and ratio of amylose: amylopectin.

In CHAPTER 5, starches with different granule size (similar composition) is hydrolyzed to understand the effect of granule size on the extent of hydrolysis (Wheat starch: large-type and small-type). Then, characterization and physicochemical properties of hydrolyzed starches were studied.

After fundamental studies, starch would be pre-treated in order to study the effect of pre-treatment on the extent of hydrolysis. Pre-treatment would be used as a pre-processing step to improve enzyme access into the starch granules, thus, increase the rate of hydrolysis. CHAPTER 6 examined the effect of heat treatment on the extent of hydrolysis for starches from different botanical origin (corn, mung bean, sago and potato). Some researcher found that heat treatment could increase or decrease the rate of hydrolysis, depending on starch type and enzyme used. Heat treatment would be conducted below gelatinization temperature (50°C) before hydrolysis in order to minimize the usage of high temperature and save energy.

CHAPTER 7 gives the study on the effect of removing surface/indigenous lipid on the extent of hydrolysis for cereal starch (wheat, corn, rice). Cereal was chosen as the starch contain higher amount of lipid compared to other sources. This is to study the action of enzyme on treated starches, focusing on cereal starch. Then, characterization and physicochemical properties of treated and hydrolyzed starches was examined.

CHAPTER 8 focuses on the effect of surface treatment (NaOH and protease-treatment) on hydrolysis of native starches. These surface treatments were able to remove protein and some lipids. The results of this experiment were expected to reveal the relative susceptibility of the granule to the surface treatments and the effect of protein removal on the degree of hydrolysis. Selected cereal starches (wheat, corn, rice) were pre-treated with NaOH and protease treatment, after which the treated starch were subjected to hydrolysis with the enzyme below gelatinization temperature.

Lastly, in CHAPTER 9, overall conclusion and recommendations for the future study of this work were provided.

CHAPTER 2

LITERATURE REVIEW

2.1 Starch

Starch can be obtained by simple processes from various plant sources such as cereal grain seeds, roots, stems, legume seeds, fruits and leaves (Hizukuri, 1996). Starch constitutes a major component of foods. The constituent macromolecules of starch are packaged in a highly ordered and compact manner, resulting in inert, insoluble granules (Oates, 1997). Starch is the major reserve polysaccharides of photosynthesis tissues and many types of storage organs such as seeds, swollen stems and roots in green plants. The outward appearance of starch granules depends on their origin and this can be easily identified under the microscope. They exhibit a variety of shapes such as round, ovoid, ellipsoid or kidney-shaped and may be flat or polyhedral.

Starch can be manufactured by the combination of grinding the starch-rich crop followed by wet separation technique. The starch granules will sediment in water due to their higher density (Chen, 2003). Native starch is white powder with bland taste and flavor, and insoluble in cold water. Starch may be chemically, enzymatically or physically modified to impart novel characteristics. The structure of starch can be described in terms of physicochemical properties of the constituent molecules, compositional variation, architecture and the macro level of the whole granule itself (Tester et al., 2004). Native starch has limited processing tolerance to heat, acid and shear. Starch is modified through cross-linking, stabilization, pre-gelatinization, conversion and others to give better processing tolerance and unique properties (Chua, 1987).

Corn (maize), wheat, rice, rye, barley and sorghum are the most common cereal starches. Tuber starches are potato, lily, canna and tulips whereas the root starches are sweet potato, tapioca (cassava), arrowroot, iris and taro. Both tuber and root starches have high water content in their reserve organs when freshly harvested (Dziedzic and Kearsley, 1984). Legume type starches include pea, mung bean and lentil (Hoover and Zhou, 2003). Starch plays an important role in human diet for its nutritional value. Starch has multifunctional uses such as gelling agent, bulking agent, anti sticky agent, water absorber, thickening agent and source of energy for fermentation in the food industries (Singh et al., 2003).

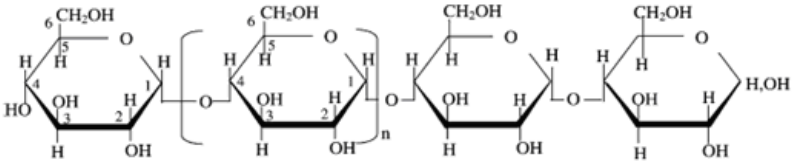
2.1.1 Starch components

Starch composes of a mixture of two distinct polysaccharides fraction – amylose and amylopectin; both consist of glucose but differ in size and shape (Oates, 1997). Both amylose and amylopectin represent approximately 98-99% (dry weight) of the starch granules (Tester et al., 2004). Amylopectin, the major component of most starches, consists of a large number of shorter chains that are bounded together at their reducing end side by a (1→6)-linkage, which makes this very large polysaccharide extensively branched (Manners, 1981). Amylose consists only of either a single or a few long chains, thus making the molecule linear or slightly branched (Takeda et al., 1992).

2.1.1.1 Amylose

Amylose is the smaller and linear fraction of the two components with the degree of polymerization (DP) around 500-5000. It posses a very small degree of branches, 9-20 per molecule and the chain length is between 4 to >100 glucose units

(Oates, 1997). Amylose (Figure 2.1) is considered to be an essentially linear polymer composed almost entirely of α-1,4-linked D-glucopyranose. The molecular weight of amylose ranging from 10^5-10^6.



Amylose: α-(1→4)-glucan; average n = ca. 1000. The linear molecule may carry a few occasional moderately long chains linked α-(1→6).

Figure 2.1: Structure of amylose (Tester et al., 2004).

Amylose molecules are thought to occur in the granule as individual molecules, randomly interspersed among the amylopectin molecules and in close proximity with one another. The location of amylose can be in the amorphous and/or crystalline region depending on the botanical source of the starch (Oates, 1997). It frequently forms a helix and also forms a very strong complex with iodine.

The amylose content of starch increases with the maturation of the seeds and the size of starch granules (Morrison and Gadan, 1987; Yoshida et al., 2003). The ratio of the amylose and amylopectin varies according to the botanical origin of the starch but mostly around 20-30% (Tester and Karkalas, 2002). However, certain mutant plants, commonly called waxy because of the waxy appearance of the seed endosperm, have a much lower content, or even lack the amylose content completely (Jane et al., 1999). Other types possess increase amylose content, called high-amylose starch (Morrison et al., 1984). According to BeMiller and Whistler (1996), wheat starch contains around 25% amylose and corn has about 25-28% amylose with the remainder being amylopectin. High amylose corn can run as high as 80% of

amylose. Rice has about 19% amylose, and waxy starches have virtually none. The ratio of amylose to amylopectin within a particular starch is a very important point to consider with respect to starch functionality in food. Their content and structure affect the architecture of the starch properties and characteristics. Amylose complexation with fats and food emulsifiers such as mono- and diglycerides can shift starch gelatinization temperatures, alter textural and viscosity profiles of the resultant paste and limit retrogradation (Thomas and Atwell, 1999).

2.1.1.2 Amylopectin

Amylopectin is the predominant molecule in most normal starches (Thomas and Atwell, 1999). It is larger than amylose with molecular weight around 1×10^7 – 1×10^9 (Biliaderis, 1998) and highly branched with the composition of 95% α- (1→ 4) linkages and 5% α- (1→ 6) linkages. There are 3 major species of amylopectin with DP_n 13400-26500, 4400-8400 and 700-2100. Compared to amylose, amylopectin have shorter chain units with a broad distribution profile. The typical range of the length is around 18-25 units on average (Hizukuri et al, 1996).

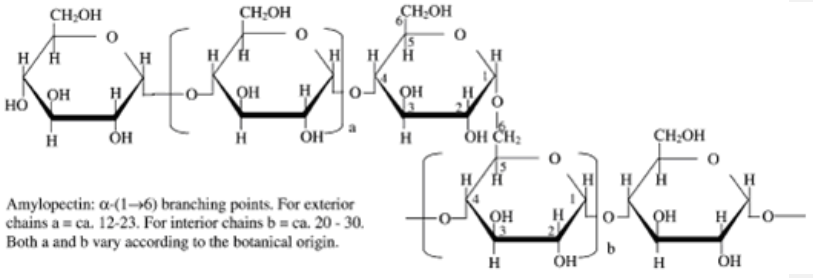


Figure 2.2: Structure of amylopectin. (Adapted from Tester and Karkalas, 2002).

The properties of amylopectin differ from those of amylose due to its highly branched nature. For example the size of the amylopectin and its three-dimensional network structure give slow retrogradation and the gel formation can be delayed or prevented. Pastes from starches that contain essentially all amylopectin (waxy starch) are considered to be non-gelling but have a cohesive and gummy texture. Furthermore, amylopectin does not give a starch-iodine (amylose-iodine) blue colour but a purple and sometimes reddish-brown colour depending upon its source. Models for structure of amylopectin are illustrated in Figure 2.2. The amylopectin molecule contains several distributions of chains differing in their chain length (Figure 2.3d). It has three types of α-1, 4 chains. The most peripheral chains are A-chains, which are un-branched and are linked to the molecule through their reducing end-group, forming α-1, 6 branches. B-chains (B_1-B_4) are joined to the molecule in the same way but carry one or more A-chains; and one C-chain, which have the reducing end-group of the molecule (Walter, 1998). Some of the physicochemical properties of amylose and amylopectin are summarized in Table 2.1.

Table 2.1: Summary of the physicochemical properties of amylose and amylopectin.

Properties	Amylose	Amylopectin
Molecular structure	Essentially linear	Highly branched
Molecular weight	150,000-400,000	10-15 million
Gels	Firm, irreversible	Soft, reversible
Films	Coherent	Not readily form
Iodine colors	Blue	Reddish-purple
Digestibility by amylase	100%	60%

(Source: Eliasson, 2004).

The most remarkable structural feature of amylopectin is it refined multiple "cluster" structure, which was proposed by French (1984). This model explained the high viscosity of amylopectin and the possibility of building the high molecular weight amylopectin (10^7-10^8). The cluster model was further developed by Manners and Matheson (1981), who claimed the branch points are arranged in "tiers" of clusters and not distributed randomly throughout the macromolecule. According to Oostergetel and van Bruggen (1989), amylopectin cluster was built up from crystalline phase with parallel glucans (crystalline lamellae) and amorphous phase with most of the branched space (amorphous lamellae).

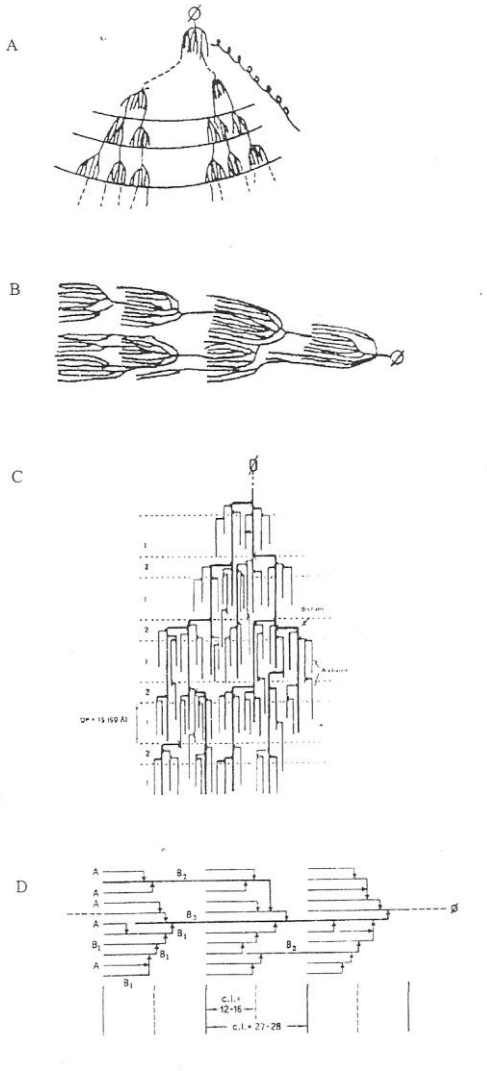


Figure 2.3: Models for amylopectin-cluster as proposed by (a) Nikuni (1969), (b) French (1984), (c) Robin et al. (1974), (d) Manners and Matheson (1981).

DP, degree of polymerization; Ø, reducing terminal.

2.1.2 Minor components of starch

Despite the presence of the major components, the existence of minor components such as lipids, proteins and minerals play a significant importance in the properties of starches. In general, cereal starches (e.g. corn, wheat, rice) contain relatively high levels of lipids (0.2% - 0.8%) and protein (0.2% - 0.5%) resulting in a lower paste transparency and persistent "raw cereal flavor" of the starch gels. Tuber (e.g. potato) and root (e.g. tapioca) starches have lower levels of lipids (0.1% - 0.2%) and protein content (0.1% - 0.2%). Potato starch is the only native starch containing significant amounts of chemically bound phosphate ester groups (degree of substitution = 0.003 ~ 0.005) located in amylopectin molecules (Table 2.1).

Table 2.2: Chemical characteristics of starch granules from different botanical sources.

Starch	Amylose (%)	Lipids (%)	Protein (%)	Phosphorus (%)
Corn[a]	28	0.8	0.35	0.00
Waxy corn[a]	<2	0.2	0.25	0.00
High amylose corn[a]	50-70	0.2	0.5	0.00
Wheat[a]	28	0.9	0.4	0.00
Potato[a]	21	0.1	0.1	0.08
Mung bean[b]	40-50	0.3	0.3	0.00

(Source: a: BeMiller and Whistler, 1996, b: Hoover et al., 1997).

2.1.2.1 Protein (nitrogen)

Protein content varied based on the source of starch. All protein analysis is reported as percent nitrogen. Proteins may exist in starches either on the surface of the granule or embedded within the matrix. Proteins can be categorized into starch surface associated proteins and integral proteins (Tester and Karkalas, 2002). "Surface" granule proteins can be extracted with salt solutions while the "integral" starch granule protein will require more vigorous extraction such as using the detergent sodium dodecyl sulphate (SDS) or an alkaline solution. The presence of protein was suspected to have a significant effect on the hardness of wheat endosperm (Baldwin, 1994).

2.1.2.2 Lipid

Lipids may be present in the form of integral lipids, which comprise of lysophospholipids (LPL) and free fatty acid (FFA) (Tester and Karkalas, 2002). LPL contains phosphate groups and this structure allows it to form a complex with amylose wherein the fatty acid group is aligned in the core of the amylose helix. This complex is very stable and dissociates only at very high temperatures (Thomas and Atwell, 1999). The complex reduces the accessibility of the active site of the enzyme making the amylose chain less likely to be hydrolyzed. There are also traces of surface lipid contaminant on the granule. The contaminants comprise of triglycerides, glycolipids, free fatty acids and phospholipids, which derived from the amyloplast membrane and non-starch sources (Tester and Karkalas, 2002).

2.1.2.3 Phosphorus

Phosphorus is found in three major forms; phosphate monoesters, phospholipids and inorganic phosphates (Blennow et al., 1998). Typically, the root (tuber) and legume sources of starch contain esterified phosphorus (phosphate monoesters). Potato starch, which consists of a high amount of phosphorus, carries a net negative charge due to the presence of this mineral. These negative charges on the individual polymer chains repel each other and these may enhance the hydration and swelling hence producing a higher viscosity (Blanshard, 1987). Most cereal starches contain very small amount of phosphorus, and if present, it is usually analyzed as phospholipids (Morrison, 1989). Phosphorus plays an extremely important role in starch functional properties such as paste clarity, viscosity, viscosity consistency and paste stability (Eliasson, 2004).

2.1.2.4 Trace elements

In addition to other compounds present in starch, starch can and does contain very small amounts of minerals and inorganic salt. During the isolation of starch products for commercial application, these compounds are assayed for and reported collectively as ‘ash’. Ash content varies on the native starches and this variance is primary dependent upon source or origin of the starch. The ash content for most commercial starches is reported to be <0.5% based on a dry starch basis (Eliasson, 2004).

2.1.2.5 Moisture (water)

Water or moisture content of starch varies significantly in its native state. Usually, starch as prepared commercially contains on the average approximately 12% moisture (Eliasson, 2004).

2.1.3 Starch granule structure

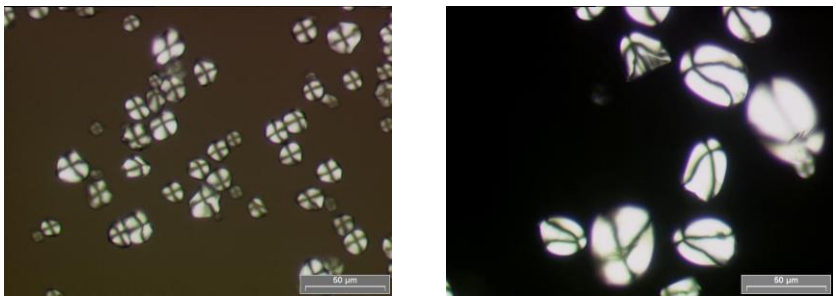
Amylose, amylopectin and other minor components such as protein, lipid and phosphorus are packed in discrete and semicrystalline aggregates called starch granules (Figure 2.4). Starch is a solid material with a density of approximately 1.5g/ml. The characteristic shape, size and morphology of the starch granules are varied depending on the different botanical source. The sizes of the granules vary from sub-micron to more than 100 microns in diameter. The shapes of granule include spherical, disk, oval, dome-shape, elongated rod shape, polygonal and compound starch (Jane, 2006).

2.1.3.1 Optical microscopy

Large hydrated granules such as potato and canna, showed the existence of "growth rings", which are shells of alternating high and low refractive index and can be observed under light microscope (Whistler et al., 1984). At the center of the granule is the original growing point or hilum. This region is usually less organized than the rest of the granule. These rings suggest that starch is deposited in a diurnal rhythm, where newly synthesized materials are deposited on the surface as the size of the granule increases (Ball et al., 1996). Due to this organization, the non-reducing ends of amylose and amylopectin radiate out towards the surface of the granule,

which allows the addition of glucose residues to extend the amylopectin chains radiating outwards (Oates, 1997).

The most effective microscope observation of native starch granules uses the polarizing microscope. Starch granules appear as distorted spherocrystals and the radial arrangement of the starch molecule displaying birefringence with the "Maltese cross" as shown in Figure 2.4. The apparent intensity of birefringence is dependent on the thickness of the granule as well as on the degree of crystallinity and orientation of the crystallites. In starch granules, the branches of amylopectin form double helices, which are arranged in crystalline domains. Contrarily, amylose largely makes up the amorphous regions, which are randomly distributed between the amylopectin clusters (Blanshard, 1987). The branching regions are constituted of the amorphous layer that separates the crystallites from each other (Eliasson and Gudmundsson 1996).



(a) Corn starch (b) Sago starch

Figure 2.4: Polarized light micrographs for (a) corn starch (b) sago starch.

Birefringence patterns obtained from the compound granules of potato starch indicated that a number of quite distinct nuclei have been formed which have subsequently grown with spherical symmetry until mutual impingement occurred.

The complex patterns exhibited by pea starches suggest that a number (from three to ten) of nuclei are formed near the center of amyloplast, and subsequently grown in a sectorial fashion. As in the case of compound potato starch, the linear interface between segments indicates that the nuclei are born simultaneously, and that crystallization proceeds at the same rate in all segments (Banks and Greenwood, 1975).

Analysis of granules with light microscopy show evidence of a layered structure in the granule, particularly for wheat, although starch from many plants only exhibits the rings or lamellae after pretreatment with acid or hydrolytic enzymes (Galliard and Bowler, 1987). However, it has been shown that starch granules have a symmetrical arrangement thought to be crystalline structure which can be seen as birefringence patterns when the granule is viewed under between the crossed polarizes. Amylose has been located in bundles between amylopectin clusters (Blanshard, 1987) and randomly interspersed among clusters in both the amorphous and crystalline regions (Jane et al., 1992; Kasemsuwan and Jane et al., 1994). The location of amylose with respect to the amorphous and/or crystalline regions is dependent on the botanical source of the starch. Large amylose molecules that are present in the granule core are able to participate in double helices with amylopectin, whereas smaller amylose molecules, present at the granule periphery, are able to leach out from the granule (Jane and Shen, 1993).

2.1.3.2 Scanning electron microscopy (SEM)

SEM can either be used to examine the gross morphology, granule ultra structure after fracture or to study variations in internal packing (Baldwin et al., 1994). SEM of undamaged or unmodified starch granules shows that the granule

surface is relatively smooth and free from pores, cracks or fissures. However, Fannon et al. (1992) reported the presence of granule surface pores randomly distributed over the surface of corn (Plate 2.1), sorghum and millet starches but concentrated at the equatorial groove of wheat, rye and barley starches. These pores rendered the granules more susceptible to enzymatic attack. No pores were found on the surface of native potato starch and legume starches. Leach and Schoch (1961) suggested that cereal starch granules have a porous structure and reported that starches from corn, waxy corn and wheat had a higher susceptibility to enzymatic attack than potato starch.

The precise pattern of hydrolysis can be followed by various microscopies, and has been found to vary between species. When a starch granules is damaged or eroded, either by acidic or enzymic treatment, the starch granules are greatly weakened so that many granules crack open and exposed a pronounce layer structure. Normally, the enzyme preferentially attacks the amorphous growth rings (Plate 2.2) and this is because the amorphous region will be attacked more readily than the semi crystalline growth rings (Eliasson, 2004).

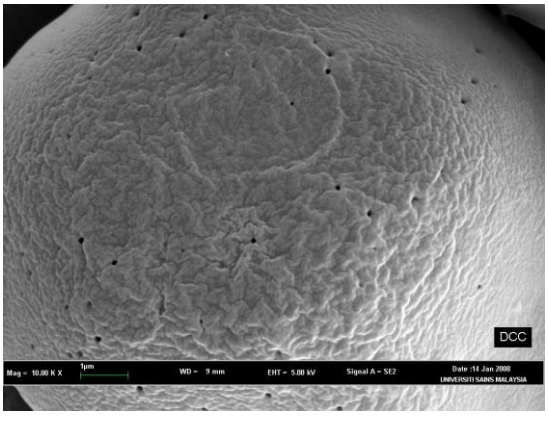


Plate 2.1: The presence of pores and pinholes on the surface of corn starch.

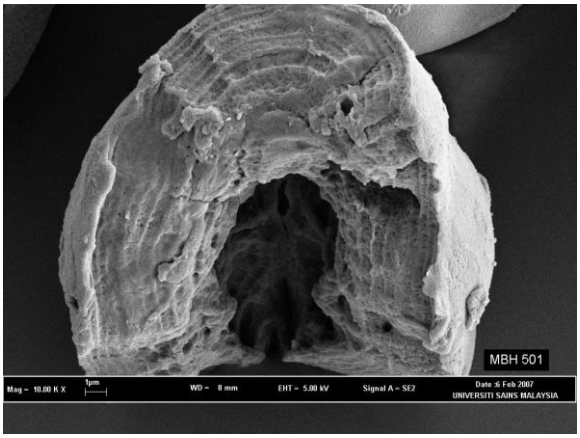


Plate 2.2: The presence of amorphous growth ring in mung bean starch after enzymatic attack.

SEM reveals that different starches are attacked in patterns that are related to the surface and internal texture of starch granules (Whistler et al., 1984). For example, if intact starch granules are exposed to the enzyme, then the first alteration in structure is seen to be pitting at the surface. In the case of corn starch, these small pits become holes, after which endocorrosion (i.e. from the inside out) appears to occur. In this case, the internal preferential loss of material from amorphous growth rings becomes clear. The granule interior may be highly eroded or solubilized, yet the exterior surface or parts of it are resistant. However, potato starch behaves differently, with substantial cracking occurring at the surface first (exocorrosion) only followed later by endocorrosion (Mathias et al., 1997).

Native starch granules are hydrolyzed much more slowly by starch hydrolyzing enzymes than a gelatinized granules or starch solution, because of its semi-crystalline nature. During hydrolysis, a range of different morphology may be observed, which range from pitting of small holes, shell formation, and surface erosion. The mode of enzymatic degradation is dependent both on the starch and the

type of enzyme employed (Planchot et al., 1995). Enzymatic attack on large wheat starch granules starts at the equatorial groove (Gallant et al., 1973). Potato starch is digested much more slowly than cereal starches. The differences in digestibility and hydrolyzed granule morphology may be connected to the presence of pores on the surface of granules and crystalline type B in potato and A in cereal starch (Eliasson, 2004).

2.1.3.3 X-ray diffraction

On the basis of X-ray diffraction studies, starch granules are said to have semicrystalline character. The amorphous regions contain the main amount of amylose, but also a considerable part of the amylopectin. The crystalline regions consist primarily of amylopectin. An internal structure of starch granules as revealed by X-ray scattering studies has been proposed (Jenkins et al., 1994) as schematically shown in Figure 2.5, i.e. (a) a single granule with alternating amorphous and semicrystalline layers, representing growth rings; (b) expanded view of the semicrystalline layer of a growth ring, consisting of alternating crystalline and amorphous lamellae.

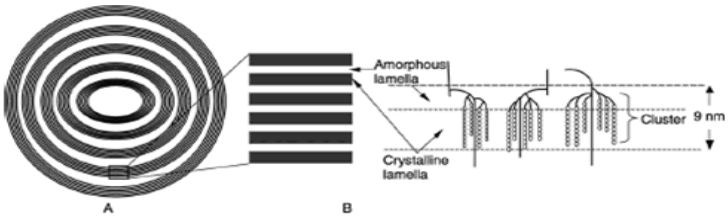


Figure 2.5: Model for the starch granule as shown by small angle X-ray scattering data (Jenkins et al., 1994).

Starch granules possess different types of crystallinity, displaying A-, B- and C-type X-ray patterns, depending on their amylopectin branch chain length (Hizukuri, 1985). Most cereal starches such as wheat, corn and rice are A-type and B-type pattern is shown by tuber, fruit and high-amylose corn starch and by retrograded starch (Tester and Karkalas, 2002). C-type pattern is an intermediate between A- and B-type starches and its typical of legume seed starches, such as pea and bean. The V-type structure has not been found in native starches, but it may form if starch recrystallizes in the presence of a fatty acid or long-chain alcohol (Hoseney, 1994). Figure 2.6 shows the X-ray diffraction patterns of A, B and C type.

The A type starch is characterized by peaks at diffraction angles 2θ of 15.3°, 17.1°, 18.2° and 23.5°. The B type X-ray pattern starch is characterized by peaks at diffraction angles 2θ of 5.6°, 17.2°, 22.2° and 24.0° whereas the C type X-ray pattern is characterized by peaks at diffraction angles 2θ of 5.7°, 15.3°, 17.3°, 18.3° and 23.5° (Zobel, 1997).

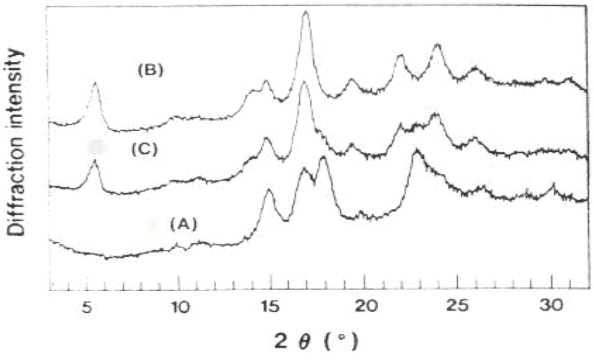


Figure 2.6: X-ray diffraction patterns of A, B and C type.
(Adapted from Hizukuri et al., 1996).

The A-type polymorphic starch has a monoclinic unit cell and the B-type polymorphic starch has a hexagonal unit cell (Imberty et al., 1991; Zobel, 1988). The C-type polymorphic starch consists of a combination of the A-type and the B-type unit cells. This classification, based on diffractometric spectra, does not follow the morphological classification but is able to group most starches conveniently according to their physical properties (Gallant et al., 1992). The additional form, V type crystallinity corresponds to structure of helical inclusion complexes of amylose occurs in swollen granules. Based on the observation made by Buleon et al. (1993), starch granules that are treated by enzymic process appears to be composed of small, almost spherical blocklets arranged tangentially. In the hard part of the shell they may be up to 100 nm in diameter, while only 25 nm in amorphous parts. B-type starches posses much larger blocklets (4-500 nm) and they are superimposed at the peripheral level to the depth of up to 10 μm (Figure 2.7). These blocklets may explain the resistance of 'B' type starches compared to 'A' type starches.

In addition, Jane (2006) had also reported that the A type starch consists of shorter branch chains whereas B-type starch consists of longer B chains. The short crystalline structure in A-type polymorphs are less stable and more susceptible for rearrangement and therefore generate more loosely packed areas of voids. On the other hand, B type polymorphs and some C type polymorphs have long chains, which extended through two or more clusters and stabilize the internal structures of granules.