# THE IMPACT OF ANNEALING, ACETYLATION, AND DUAL MODIFICATION ON THE STRUCTURE AND PHYSICOCHEMICAL PROPERTIES OF WAXY STARCHES

by

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## ABSTRACT

Physical and chemical modification techniques are often used to modify native starch to enhance its positive attributes which are required in industrial processes. The present study focused on modification of waxy starches using annealing (ANN), acetylation, and dual modification (ANN-acetylation) and to evaluate their impact on the morphology, molecular structure and properties of waxy corn (WC), waxy barley (WB), waxy rice (WR) and waxy potato (WP) starches. In addition, the uses of methylation and mass spectrometry analysis helped to unravel the structures of native and modified starches. Acetylated starches were prepared using different levels of acetic anhydride (5, 10, and 20%) and dual modification was achieved by ANN for 72 h, followed by acetylation (10%). Scanning electron microscopy showed that granular morphology did not change upon ANN, but granular surface became rough and slight fragmentations, and indentations were observed upon acetylation and dual modification. In the $^{1}$H-NMR spectra, the characteristic peaks for acetyl protons (2.01-2.06 ppm) were detected. The introduction of acetyl groups was confirmed by FTIR spectroscopy with new absorption bands appearing at around 1737, 1375, 1252 $cm^{-1}$. X-ray diffraction pattern remained unchanged upon modification. The relative crystallinity of annealed starches remained unchanged except for WC, while acetylation and dual modification decreased relative crystallinity. Gelatinization temperatures increased on ANN and dual modification but decreased on acetylation. The gelatinization enthalpy remained unchanged upon ANN but decreased upon acetylation and dual modification. Acid hydrolysis decreased in WP, but remained unchanged in other starches on ANN. *In vitro* digestibility did not significantly change upon annealing except for WP however, it significantly decreased upon acetylation. Acetylation decreased RDS and SDS contents but increased that of RS. The dual modification resulted in lower *in vitro* digestibility and increased the RS content to a similar or

higher extent than that of 20% acetic anhydride treatment, except for WR. Retrogradation characteristics were affected by modifications. Annealing increased light transmittance in WC and WR, while it decreased in WP. However, the impact of ANN on retrogradation endotherms was only marginal. Acetylation decreased retrogradation as evidenced by increased light transmittance and lower retrogradation enthalpy. The increase in light transmittance of dual modified starches was higher than acetylated starches with 10% acetic anhydride and the decrease in retrogradation enthalpy was significantly lower ($p<0.05$) than that of 5% acetic anhydride treatment in WC and WP, and retrogradation endotherms were not observed in dual modified WR and WB. ESI-MS analysis of permethylated native starches resulted in a series of sodiated molecular ions and low energy collision induced dissociation tandem mass spectrometry analysis (CID-MS/MS) indicating glycosidic and cross ring fragmentations of the precursor sodiated molecular ions. ESI-MS analysis of hydrolyzed acetylated and dual modified starches resulted in oligomers with various substitutions (1-3 acetyl groups). Furthermore, ESI-MS/MS analysis showed that when there were two acetyl groups, those were not located in the same glucose molecule, but were located in two different glucose molecules. The results of this study demonstrated that dual modification has a promising effect on improving functionality in waxy starches, and that tandem mass spectrometry can be used to study the structure of chemically modified starches.

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## LIST OF ABBREVIATIONS

ADP - Adenosine diphosphate

AM - Amylose

ANN - Annealing

AP - Amylopectin

APCLD - Amylopectin chain length distribution

CID - Collision induced dissociation

$\overline{CL}$ - Chain length

db - Dry basis

DMSO - Dimethyl sulphoxide

DP - Degree of polymerisation

DSC - Differential scanning calorimetry

DS - Degree of substitution

ESI - Electrospray ionization

FACE - Fluorophore-assisted carbohydrate electrophoresis

FTIR - Fourier transform Infrared spectroscopy

$^{1}$H NMR - Proton nuclear magnetic resonance

HPAEC-PAD - High performance anion exchange chromatography with pulsed amperometric detection

MALDI - Matrix-assisted laser desorption/ionization

MS - Mass spectrometry

RDS - Rapidly digestible starch

RS - Resistant starch

SBE - Starch branching enzyme

SDS - Slowly digestible starch

SEC - Size exclusion chromatography

SEM - Scanning electron microscopy

SS - Starch synthase

WAXS - Wide angle X-ray scattering

# CHAPTER 1

# INTRODUCTION AND OVERVIEW

Waxy starches consist almost exclusively of amylopectin and are produced either by traditional plant breeding techniques or biotechnological means. Genetically modified waxy starches are produced by the simultaneous antisense downregulation of three starch synthase genes (Granular Bound Starch Synthetase, Starch Synthetase II, and Starch Synthetase III) (Neelam, Vijay &Lalit, 2012). Although, waxy corn starch had previously gained more attention in the scientific research and in commercial applications, the production and use of other waxy starches such as, waxy barley, wheat, sorghum, potato, and rice have recently increased (Šárka & Dvořáček, 2017). Waxy starches gelatinize easily, produce clear gels, and their retrogradation is slower, hence, they can be used in many food and non-food applications.

The industrial utilization of native starch is limited due to the inherent imperfect nature, such as water insolubility, low stability at low pH, high temperature, and tendency to easily retrograde and undergo syneresis forming unstable pastes and gels (Ashogbon & Akintayo, 2014). Starch modification techniques (physical, chemical, enzymatic, biotechnological, or their combinations) have been used to produce various novel derivatives with improved physicochemical properties that can be used in industrial applications. In recent years, there has been intense research to develop new methods of starch modifications by placing more emphasis on physical and chemical modifications (Ashogbon & Akintayo, 2014).

Annealing is a hydrothermal modification technique which alters physicochemical properties of starch without destroying the granule structure. Annealing is the physical treatment of starch at low temperature (5-10 $^{o}$C below the onset temperature of gelatinization) and high moisture

content. The aim of annealing is to approach the glass transition temperature ($T_g$), which facilitates molecular mobility without triggering gelatinization (Neelam et al., 2012). $T_g$ refers to the temperature at which the amorphous domains of the starch granule are transformed from a rigid glassy to a mobile rubbery state when heated in the presence of solvents such as water or glycerol (Jayakody & Hoover, 2008). Crystalline perfection and increased interaction between amylose-amylose, amylopectin-amylopectin, and amylose-amylopectin have been attributed to the changes in functionality in annealed starches such as decreased granule swelling and amylose leaching, increased gelatinization transition temperatures and thermal stability (Chung, Hoover, & Liu, 2009a).

Chemical modification involves the introduction of functional groups into the starch molecules, which alters their physicochemical properties. Acetylation is a chemical modification of starch in which starch hydroxyl groups are substituted with acetyl groups (Neelam et al., 2012). Acetylation causes changes in the gelatinization, pasting, and retrogradation behavior of starch since introduction of acetyl groups reduces the bond strength between starch molecules and minimizes association between starch molecules. A low degree of substitution (DS) with 0.01–0.2 acetylated starch has been applied in many food applications, whereas high DS acetylated starches are used in non-food applications (Ashogbon & Akintayo, 2014).

Both physical and chemical modifications of starch have advantages as well as limitations. Even though chemical modification improves functionality of starch, there is a general disapproval for chemically modified starch among consumers. Combining physical and chemical modification techniques (dual modification) would provide new insights to produce starch with desired functionality using minimal chemicals. Dual modification of starch has been reported in literature which involves two types of chemical, physical, and chemical and physical

modification techniques (Varavinit, Paisanjit, Tukomane, & Pukkahuta, 2007; Deetae et al., 2008; Chung et al., 2009a).

Even though a substantial number of studies have been done to investigate the impact of annealing and acetylation, the results are contradictory due to variations in the starch source, annealing and acetylation conditions, and the methods used to determine structural and physicochemical properties (Waduge et al., 2006; Lan et al., 2008; Wang et al., 2014; Zeng et al., 2015). Further, the information on topochemical aspects of acetylated starches is scare. Detailed information on substitution can lead to better understanding of the relationships between molecular structure and functional properties of modified starches. Most of the studies have been focused on starches with amylose content in the range of 25-35%. There is a dearth of information on the impact of annealing and acetylation as well as annealing-acetylation as a dual modification method on starches devoid of amylose (waxy starches). Hence, we hypothesized that changes in the structure of starches which are devoid of amylose on annealing, acetylation, and dual modification could vary depending on the structure of native starch. Particularly in acetylation and dual modification, structural changes would depend on substitution distribution, and these changes would affect the properties of starch. Furthermore, we hypothesized that mass spectrometry would help with identification of the distribution of these substitute. Thus, the objectives of this study were; 1) to determine the effect of annealing, acetylation and annealing-acetylation (dual modification) on the structural organization of starch chains within the amorphous and crystalline domains and physicochemical properties of waxy starches from different botanical origins, namely -waxy corn, waxy rice, waxy barley, and waxy potato, and 2) to investigate the substitution distribution of acetylated and annealing-acetylated waxy starches using mass spectrometry.

# CHAPTER 2
# LITERATURE REVIEW

## 2.1 Starch

Starch is a semicrystalline biopolymer which is produced in higher plants. It is stored in seeds, fruits, stems, roots, and tubers in most plants, in the form of granules. A greater proportion of the energy available to non-photosynthetic organisms comes from starch, which is found in the principal food crops of the world, mainly cereal grains (wheat, rice, maize, barley, rye, sorghum), tubers (potato, sweet potato, yam), root crops (tapioca, arrowroot, taro) and seeds of legumes (peas and beans). Starch provides 50-70% of the energy requirement in the human diet, serving as a direct source of glucose. It serves as an important material for both food and non-food industries (Srikaeo, 2016).

Approximately, 60 million tonnes of starch are extracted annually from cereal, root and tuber crops, of which 60% is used in the food industry and 40% in pharmaceuticals and other non-food industries (Burrell, 2003; Copeland, Blazek, Salman & Tang, 2009). However, native starches have poor functionality (low thermal and shear resistance, susceptibility towards retrogradation and syneresis), and therefore, they are often modified chemically and/or physically to enhance their functional properties relevant to industrial applications (Jayakody & Hoover, 2008; Chiu & Solarek, 2009). Different industrial applications of native and modified starches are given in Table 2.

Waxy starches are used in many food applications industrially because of their paste clarity and viscous stability and slow retrogradation properties. Waxy starches have been modified using various processing techniques to increase resistant starch content. These processes include

debranching, encapsulation, extrusion cooking, hydrothermal treatment, chemical modification, and enzymatic modification (Šárka & Dvořáček, 2017). Waxy rice is widely used in Asian countries (China and Korea) in snacks, soups, and gravies due to its stickiness characteristic, more porous texture, and good water retention (Bao, Corke, & Sun, 2004). Waxy corn starches are widely used as thickeners, binders, stabilizers, and food additives (Lu et al., 2014). Waxy corn has been used to produce starch nanocrystals which have gained considerable attention as novel and biofunctional materials in diverse industries. These nanocrystals have potential use as carriers for drug delivery and stabilizers in oil-in-water emulsions (Šárka & Dvořáček, 2017). Waxy wheat is used in baked foods due to its better processing properties such as water-holding, dough development time, extensibility, swelling power and setback (Zhang, Zhang, Xu, & Zhou, 2014). Waxy barley is cultivated in North America because its flour confers freeze-thaw and anti-staling properties to processed food (Šárka & Dvořáček, 2017).

**Table 2.1** Industrial applications of starches

| Application | Uses of native/modified starches |
|---|---|
| Food | Bakery products, infant foods, snacks, confectioneries, frozen foods, favor encapsulations, dairy products, canned foods, beverages,syrups |
| Pharmaceutical/Medical | Pharmaceutical excipient, tablet superdisintegrant, controlled/sustained release polymer, plasma volume expander |
| Cosmetics | Face and talcum powders, soap fillers/extenders, face cream, tooth paste |
| Detergent | Surfactants, builders, co-builders, bleaching agents and bleaching activators |
| Agrochemicals | Pesticide delivery, seed coating |
| Paper | Binding and coating |
| Textile | Warp sizing, fabric printing and finishing |
| Plastic | Biodegradable raw material and filler |
| Adhesive | Book binding, laminating, wood adhesives, hot-melt glues |
| Petroleum/oil drilling | Oilfield applications such as filtrate-loss control, mud-rheology modification and shale stabilization |
| Fuel | Bioethanol |
| Other | Ceramics, concrete, flocculent for waste water treatment, explosives |

Adapted from Ellis et al. (1998), Davis, Supatcharee, Khandelwal, and Chibbar (2003), Singh, Nath and Singh (2010).

## 2.2 Biosynthesis of amylose and amylopectin

Starch biosynthesis is a complex process (Tester, Karkalas, & Qi, 2004). The biosynthesis of starch involves not only the production of the composite glucans but also their arrangement into an organized structure within the starch granule (Martinn& Smith, 1995). Starch is synthesized in the plastid of both photosynthetic and non-photosynthetic cells in higher plants. In leaves, transitory starch is synthesized in chloroplasts during photosynthesis and degraded at night to provide substrates for leaf respiration and for continued sucrose synthesis for export to the rest of the plant. In non-photosynthetic organs (e.g., stems, roots, tubers, and seeds), sucrose may be converted to starch for long term storage in amyloplasts (Zeeman, Kossmann, & Smith, 2010). Starch biosynthesis steps involve three committed enzymes: ADP-glucose pyrophosphorylase (ADPGPPase), starch synthase (SS), and starch branching enzyme (SBE). Both amylose and amylopectin are synthesized from ADP-glucose. The reaction that converts glucose-1-phosphate and ATP to ADP-glucose and pyrophosphate is catalyzed by ADPGPPase (Martin & Smith, 1995). Higher-plant starch synthases are classified into five distinct classes: granule-bound starch synthase (GBSS), and soluble SSI to IV. GBSS is responsible for amylose synthesis and other soluble starch synthases generate the chains in amylopectin (Geigenberger, 2011). SS catalyzes the synthesis of linear glucan chains by adding a glucose moiety from ADP-glucose to the non-reducing end of an α-D-(1→4) linked glucan chain, causing the release of ADP (Martin & Smith, 1995). Mutants and transgenic plants lacking GBSS enzyme are essentially amylose free (Denyer, Johnson, Zeeman, & Smith, 2001). Amylose-free starches are known as “waxy” starches due to the waxy appearance of the endosperm tissue (Alcázar-Alay & Meireles, 2015). Branching of amylopectin is catalyzed by branching enzymes, which cut an existing α-D-(1→4) glucan chain and join the cut segment of six or more glucose units to the C6 position of a

glucosyl residue of another (or the same) glucan chain by an α-D-(1→6) linkage (Zeeman et al., 2010). SBEs are classified into two classes (SBE I and SBE II) and SBE I preferentially transfer longer chains than SBE II (Tomlinson & Denyer, 2003). Amylopectin synthesis also involves debranching enzymes, which cleave branch points and are important in tailoring the structure of amylopectin. Isoamylase and limit dextrinase are the two types of debranching enzymes, which differs by their amino acid sequences and substrate specificities (Geigenberger, 2011). Recent genetic and biochemical evidences indicate that the different isoforms of SS, SBE, and debranching enzyme probably play specific roles in determining the complex starch structure and starch synthesis is achieved through the coordinated interactions of these enzymes (Tetlow et al., 2004).

**2.3 Starch granule morphology**

Starch granule size and shape are varied depending on the botanical origin (Jane, Kasemsuwan, Leas, Zobel, & Robyt, 1994). Even though most starch granules are synthesized individually in separate amyloplasts, occasionally, more than one granule is synthesized simultaneously in a single amyloplast, and these granules occurred as compound granules (Pérez & Bertoft, 2010). The size of starch granules vary from submicron to more than 100 microns in diameter. The granule shapes of starch include spherical, oval, polygonal, lenticular, elongated, disc, kidney shapes and compound starch (Jane, 2006; Pérez & Bertoft, 2010). Light microscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), atomic force microscopy (AFM) and confocal laser scanning microscopy (CLSM) are widely used to characterize starch granule morphology (Jane et al.,1994; Gallant, Bouchet, & Baldwin, 1997; Glaring, Koch, & Blennow, 2006; Chen et al., 2009).

Tuber and root starch granules are mostly oval, although round, polygonal, spherical, and irregular shapes are occasionally found, with size ranging from 1 to 110 μm depending on the starch source (Hoover, 2001). Cereal starches are generally small and polygonal in shape (Emmambux & Taylor, 2013). In some cereal starches, specifically barley and wheat, two populations of granules can be distinguished by size; large A-type granules (>10μm) and small B-type granules (<10μm) (Geera, Nelson, Souza, & Huber, 2006). Pulse starches have different shapes including oval, spherical, round, elliptical, and irregular with sizes ranging from 5 to 55 μm in width and 5 to 70 μm in length (Hoover, Hughes, Chung, & Liu, 2010). High amylose maize starch granules are often elongated and contain filamentous structures (Glaring et al., 2006). General characteristics of starch granules from different botanical sources are presented in Table 2.2.

Starch granule surface can be smooth or have pores, channels and cracks. Surface pores have been observed in some cereal starches including corn, sorghum, and millet, while equatorial grooves were found in large granules of barley, rye, and wheat. However, no pores were observed in rice and oat, which have compound granules (Fannon, Hauber, & BeMiller, 1992). These pores are natural features of the native granule structure which might be the sites of initial enzyme attack and are notartifacts produced by the isolation, specimen preparation or observation techniques (Fannon et al., 1992; Gallant et al., 1997). Channels were observed at varying frequencies in wheat, barley, and maize granules. Frequent internal cracks were observed in waxy maize compared to high amylose potato, indicating the role of amylose in determining starch granule integrity (Glaring et al., 2006).

**Table 2.2** General characteristics of starch granules

| Starch source | Shape | Size (μm) | Reference |
|---|---|---|---|
| **Cereal** | | | |
| Maize (waxy, normal, & high amylose) | Spherical, polyhedral | 3-20 | Wang et al. (2014) |
| Rice | Polyhedral, round | 2.4-5.4 | Sodhi and Singh (2003) |
| Waxy rice | Polyhedral | 5-6 | Tatongjai and Lumdubwong (2010) |
| Wheat | Oval to round (A-type) | >10 | Tao et al. (2016) |
| | Oval to round (B-type) | <10 | |
| Barley | Disk shape (A-type) | 10-35 | Ao and Jane (2007) |
| | Spherical (B-type) | 2-10 | |
| **Tuber and root** | | | |
| Potato | Oval to spherical | 10-110 | Gunaratne and Hoover (2002) |
| Waxy potato | Rounded,oval | 14-44 | McPherson and Jane (1999) |
| True yam | Oblong to oval | 12-100 | Gunaratne and Hoover (2002) |
| Taro | Round to variable | 10-50 | Gunaratne and Hoover (2002) |
| New cocoyam | Polygonal to variable | 3-10 | Gunaratne and Hoover (2002) |
| Cassava | Round to variable | 5-45 | Gunaratne and Hoover (2002) |
| **Pulse** | | | |
| Black bean | Oval/round/spherical | 7-30 | Hoover and Ratnayake (2002) |
| Chick pea | Oval/spherical | 9-30 | Hoover and Ratnayake (2002) |
| Navy bean | Oval/round/elliptical | 14-32 | Hoover and Ratnayake (2002) |
| Smooth pea | Oval/round/irregular | 2-50 | Ratnayake et al. (2001) |
| Kidney bean | Oval/round/elliptical | 5-35 | Yoshida et al. (2003) |
| Lentil | Oval/round/elliptical | 6-37 | Hoover and Manuel (1995) |

## 2.4 Molecular architecture of starch

Most starch granules, regardless of the plant origin or tissue, comprise two principal types of alpha glucans, amylose and amylopectin, which represent approximately 98–99% of the dry weight (Tester et al., 2004). The ratio of the two polysaccharides differs according to the botanical origin of the starch. In “normal” starches, amylopectin constitutes the major component by weight, while amylose constitutes 15–30%. Waxy starches contain no or very little amylose (Pérez & Bertoft, 2010). Genetically modified high amylose starches may contain 50–80% amylose (Shi, Capitani, Trzasko,& Jeffcoat, 1998).

The structure of starch in a grain can be categorized into six different length scales (Fig. 2.1): 1) individual branches of amylose and amylopectin, which are often termed as chain length distribution (1 nm) (Dona, Pages, Gilbert, & Kuchel, 2010), 2) whole starch molecules which represent the structure of heavily branched amylopectin that comprises A chains, which have no branches and are connected to a B or C chain through an α-D-(1→6) bond, B chains that have branches and connected via an α-D-(1→6) bond, and a C chain that is the backbone of the amylopectin molecule with a free reducing end (Peat, Whelan, & Thomas, 1956), 3) lamellar structure (9 nm) which composed of crystalline lamellar containing amylopectin double helices and amorphous lamellar comprising branch points (Jacobs & Delcour, 1998), 4) starch granules with concentric growth rings which are 120-400 nm in thickness, contain alternating crystalline and amorphous regions (Tester et al., 2004), 5) endosperm which comprises the starch granules together with protein and lipid, and 6) whole grain with approximately 1 mm in size and includes highest level of structures such as hull (Dona et al., 2010).

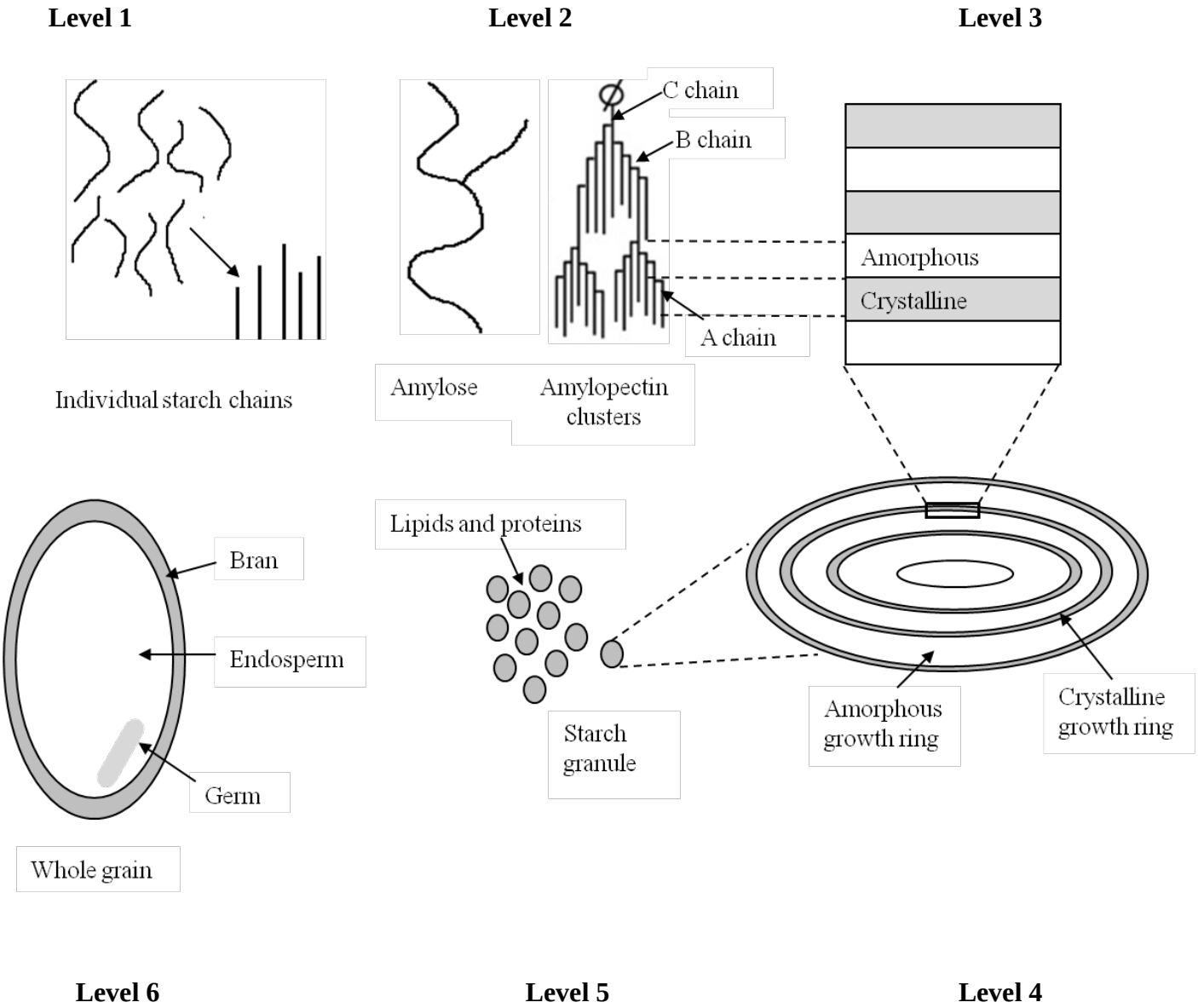



**Figure 2.1** Six supramolecular levels of the rice grain, highlighting the microscopic structural contribution of starch (Adapted from Dona et al., 2010)

## 2.5 Amylose

### 2.5.1 Structure of amylose

Amyolse is a relatively long, linear molecule with α-D-(1→4) linkage (Fig 2.2). However, slightly branched amylose molecules also exist. The molecular size of amylose varies depending on the botanical origin. In general, linear amylose molecules are low in molecular weight than their branched counterparts (Takeda, Hizukuri, Takeda, & Suzuki, 1987). The definite unit chain distribution in branched amyloses is not known, since the components cannot be separated from the linear portion (Pérez & Bertoft, 2010). However, linearity of amylose was examined with susceptibility towards the exo-acting enzyme β-amylase, which hydrolyses α-D-(1→4) linkage from the non-reducing end of the polysaccharide chains releasing β-maltosyl units, but cannot cleave the α-D-(1→6) bonds (Buleon, Colonna, Planchot, & Ball, 1998). All the branched amylose is reduced into β-limit dextrins (β-LD) that contain all branches and the residual internal chain segments. The degree of polymerization (DP) by number($DP_n$) of the β-LD of amyloses from different sources range from 700 to 2000 and the average chain length (CLn) from 50 to 160 (Takeda et al., 1987; Takeda, Maruta, & Hizukuri, 1992; Pérez & Bertoft, 2010). The molecular weight of amylose is approximately $1\times 10^5$- $1\times10^6$ (Biliaderis, 1998; Buléon et al.,1998) and the $DP_n$ is 324–4920 with around 9–20 branch points equivalent to 3–11 chains per molecule (Morrison & Karkalas, 1990; Hizukuri, 1993;Yoshimoto,Tashiro, Takenouchi, & Takeda, 2000). Each chain contains approximately 200-700 glucose residues equivalent to a molecular weight of 32,400- 113,400 (Morrison & Karkalas, 1990; Tester & Karkalas, 2002).

Amylose occurs in double-helical A- and B-amyloses and the single-helical V-amylose. Both A and B structures are left-handed helices with six glucose units per turn and differ in the packing of the starch helices (Buléon et al., 1998). A minimum chain length of DP 10 is required for

double helix formation in a pure oligosaccharide solution (Gidley & Bulpin, 1987; Pfannemuller, 1987). Single amylose chains have a unique ability to form complexes with a variety of complexing agents such as iodine, dimethyl sulfoxide (DMSO), alcohols or fatty acids and form V-type single helices. Depending on the size of these complexing agents, the amylose chain can take up a helical structure having either six, seven or eight glucose units per turn (Pérez & Bertoft, 2010). The helix possesses a hydrophobic cavity which provides binding sites with a high affinity for the non polar (part of the) ligand (Whittam et al., 1989). The formation of single helix was induced by the lower energy of the ligand in the helix cavity and the driving force for the helical formation was attributed to the tendency of amylose to minimize its interaction with water (Heinemann, Conde-Petit, Nuessli, & Escher, 2001; Putseys, Lamberts, & Delcour, 2010). Theoretically 18-24 glucose units are required for the complexation of one lipid molecule (i.e. fatty acid or monoacylglycerol with 14, 16, 18,… carbon atoms in its tail), organized in three turns, each containing six or eight glucose residues per pitch (Fig 2.3) (Carlson, Larsson, Dinh-Nguyen, & Krog, 1979). The structure of amylose-fatty acids inclusion complexes has been intensively studied by X-ray diffraction (XRD), nuclear magnetic resonance(NMR), and differential scanning calorimetry (DSC), and to some extent by small-angle X-ray scattering (SAXS), transmission electron microscopy (TEM), and Raman spectroscopy (Kong, Yucel, Yoksan, Elias, & Ziegler, 2018). When ligands, specifically lipids, are added to a starch system, complexes can be formed *in situ* affecting starch properties, such as gelatinization, viscosity, gelation, retrogradation and hydrolysis strongly (Putseys et al., 2010).

The conformation of amylose in solution has long been a matter of interest for the scientists. Three molecular models, namely a random coil with no helical structure, an interrupted helix with alternate coil portions and helical sequences, and a continuously bending helix have been

proposed based on the data analysis of conformation-dependent properties in aqueous solvents and DMSO (Norisuye, 1996). The amylose chain adopts an unstable random coil structure in a freshly prepared aqueous solution (Hayashi, Kinoshita & Miyake, 1981), whereas the helical conformation is achieved in neutral or alkaline solutions with complexing agents (lipids, iodine) (Banks & Greenwood, 1971). Interrupted helix conformation comprises regions of loose and extended helices, which alternate with shorter random coil segments (Senior & Hamori, 1973).

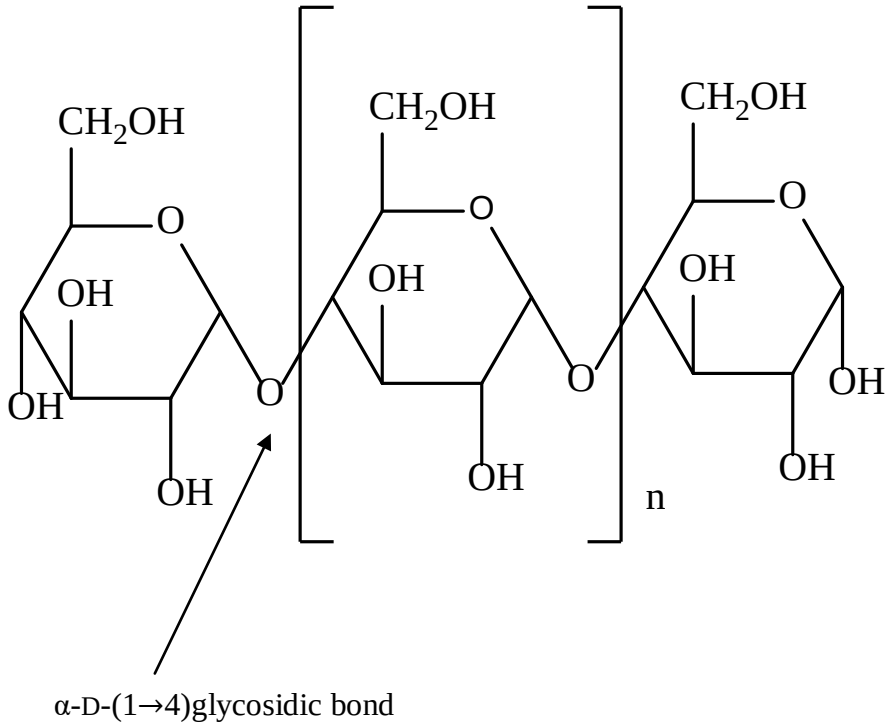



**Figure 2.2** Schematic diagram of amylose

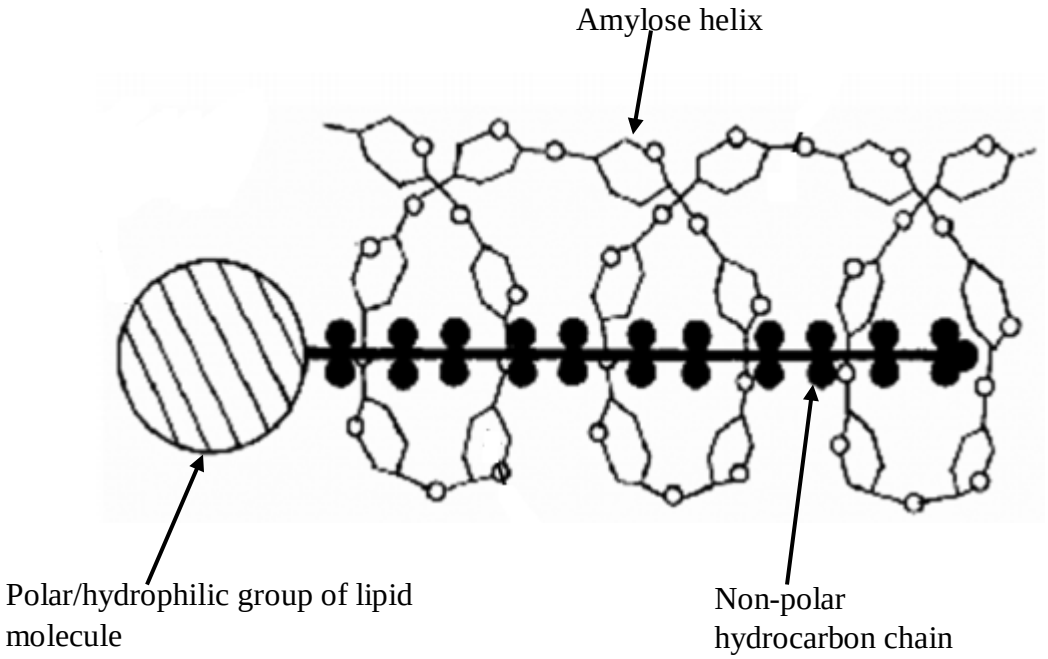



**Figure 2.3** Schematic representations of amylose-lipid complex (Adapted from Carlson et al., 1979)

### 2.5.2 Location of amylose

The actual localization of amylose within the granules remains a matter of debate, even though it is thought to be present primarily in the amorphous region (Jayakody & Hoover, 2008). Jane and Shen (1993), using surface gelatinization studies in the presence of calcium chloride, have shown that in corn and potato starches, amylose is more concentrated at the periphery than at the core of the granule. In contrast, Blennow et al. (2003), using confocal laser scanning microscopy (CLSM) and 8-amino-1,3,6-pyrenetrisulfonic acid (APTS) as a fluorescent probe for reducing ends, showed that amylose is more confined to the interior parts of granules in potato, tapioca, maize, wheat, barley, and pea starches.

Another intriguing question is whether amylose is dispersed among amylopectin or is separated from amylopectin and present in bundles (Jane, 2006; Bertoft, 2017). Based on various properties of starch granules such as amylose leaching, amylose-iodine complexation, DMSO solubilization and V-complex formation, amylose is proposed of being separated from amylopectin in the case of normal maize starch and being interspersed with amylopectin in potato starch (Zobel, 1988). Whereas, cross-linking studies of corn and potato starches have shown that amylose is interspersed among amylopectin molecules (Jane, Xu, Radosavljevic, & Seib, 1992). Based on small angle X-ray scattering studies on maize starches of varying amylose contents, it was shown that amylose disrupts the packing of amylopectin double helices within the crystalline lamella (Jenkins & Donald, 1995). Further, the studies on maize, pea, and barley starch granules indicated that amylose molecules disrupt the structural order of amylopectin clusters (Atkin, Cheng, Abeysekera, & Robards, 1999). Enzyme gold-labelling study has shown the presence of alternating layers of densely packed amylopectin and amylose molecules in low amylose potato starch. However, in amylomaize starch, granules were shown to possess an

amylopectin center surrounded by an amylose periphery encapsulated by an amylopectin surface (Atkinet al., 1999). Several studies have shown that in potato starch, amylose is co-crystallized (Fig 2.4) with the outer branches of amylopectin chains, forming double helices (Hoover & Vasanthan, 1994; Jenkins & Donald, 1995; Saibene & Seetharaman, 2010).

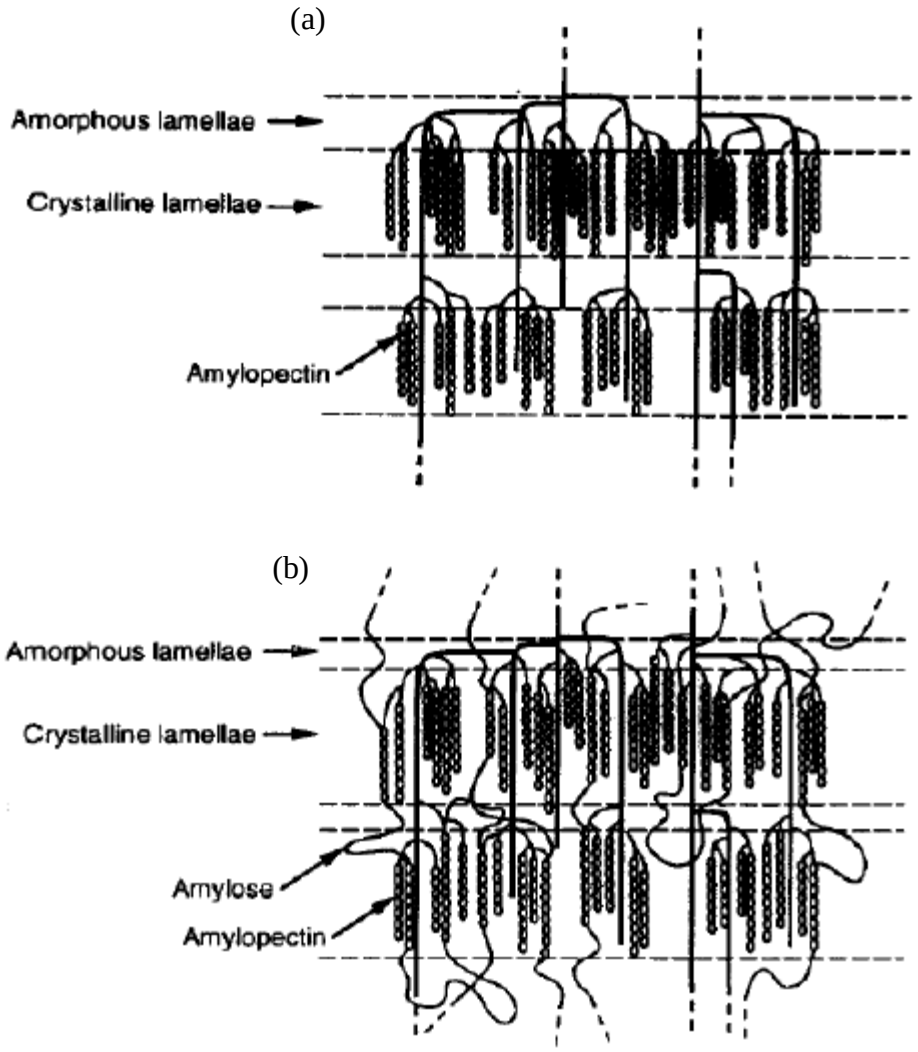



**Figure 2.4** Mechanism explaining the disruption of amylopectin double helical packing by amylose: (a) Amylopectin structure with no amylose present (b) Co-crystallization of amylose with amylopectin pulls a number of amylopectin chains out of register (Jenkins & Donald, 1995, Copyright Elsevier, reproduced with permission).

## 2.6 Amylopectin

### 2.6.1 Structure of amylopectin

Amylopectin is the highly branched component of starch which is built from mainly α-D-(1→4) linkages and 5-6 % of α-D-(1→6) linkages at the branch points (Fig 2.5) and has a molecular weight of $1\times10^{7}$-$1\times10^{9}$ (Morrison & Karkalas, 1990; Buléon et al. 1998; Tester et al., 2004). Amylopectin from various sources comprises three major species with $DP_n$ ranges of 13,400–26,500, 4400–8400 and 700–2100, but $DP_n$ of 9600- 15,900 generally predominates (Takeda, Shibahara, & Hanashiro, 2003).

#### 2.6.1.1 Unit chain distribution of amylopectin

Amylopectin consists of numerous chains that are relatively shorter than the major chains found in amylose. Peat, Whelan, and Thomas (1952) defined the organization of chains in amylopectin: unsubstituted outer A-chains, B-chains which bear other chains as branches, and C-chain which carries other chains as branches but comprises the sole reducing terminal residue of the amylopectin molecule. The B-chains can be subdivided into $B_1$- $B_4$ based on the average DP (Hizukuri, 1986). The chain lengths of A, $B_1$ to $B_4$ chains of amylopectin from various starch sources have been shown to be in the range of 12-16, 20-24,42-48,69-75 and 101-119, respectively (Hizukuri, 1986; Wang & White, 1994). Hanashiro, Tagawa, Shibahara, Iwata, and Takeda (2002) described the distribution of C-chains after labeling with a fluorescent dye and found that chains covered the DP range 10–130, possessing a peak in size-exclusion chromatograms at DP 40 for several amylopectin samples. The A and $B_1$ chains are the most external (exterior) and form double helices (and crystallites) within the native granules (Tester et al., 2004). The ratio of A-chains to B-chains, which is also referred to as the degree of multiple

branching, is an important parameter. Generally, amylopectins have more A-chains than B-chains, with ratios ranging from 1.0:0 to 1.5:1(Buléon et al., 1998).

The size distribution of amylopectin chains is analyzed after debranching, by either size exclusion chromatography (SEC), high performance anion-exchange chromatography (HPAEC) or fluorophore-assisted carbohydrate electrophoresis (FACE) (Bertoft, 2017). F1 (long B chains) and F2 (short A and B chains) fractions were separated by SEC showing the bimodal distribution of amylopectin chain lengths, but SEC could not separate individual chains. HPAEC with pulsed amperometric detection (HPAEC-PAD) was able to separate amylopectin chains over DP 80, hence, frequently used to analyze amylopectin chain length distribution(Hanashiro, Abe, & Hizukuri, 1996). Yao, Guiltinan, and Thompson (2005) compared high performance size exclusion chromatography (HPSEC) and FACE to analyze amylopectin chain length distribution and concluded that HPSEC has the capability to quantitatively describe the chains longer than DP 50, while FACE has no such capability. However, FACE has the advantage in more precisely quantifying the short chains (DP 6- 11) (Yao et al., 2005).

#### 2.6.1.2 Organization of structural units in amylopectin

The organization of chains in the amylopectin molecule has been described by different models and currently two major models have gained interest: cluster model and the building block backbone model. The principal difference between the backbone and traditional models is the completely different visualization of chain organization within amylopectin (Chauhan & Seetharaman, 2013). The cluster model proposes that the chains are not linked randomly, rather, the short unit chains (outer A-chains and inner B-chains with less DP) are organized into clusters and the long chains interconnect them (Fig 2.6) (Hizukuri, 1986; Buléon et al. 1998).

The building block backbone model (Bertoft, 2004) suggests that the clusters are built up from still smaller structural units called building blocks (Fig 2.7). It is suggested that the branches are not evenly distributed within the clusters but found in small groups which are known as building blocks (Pérez & Bertoft, 2010). These units consist of up to 10 or more chains with internal chain length (ICL) of only 1–3 residues (Bertoft, Koch, & Åman, 2012). B-chains in amylopectin are divided to external and internal segments, where the average external chain length (ECL) is between 10.7 and 15.0, depending on the starch sample, and the average ICL is defined as the segments between branches, which is only 4.6–8.0. The internal unit B-chains of amylopectin are in general categorized into two major groups of long and short chains and these short chains are further separated into two subgroups of which one is a major group (DP 8-25) and the other, minor group (DP 3-7), known as "fingerprint" B-chains (Bertoft, Piyachomkwan, Chatakanonda, & Sriroth, 2008). The building blocks were suggested to be outspread along a backbone consisting mostly, but not entirely, of the long unit chains in amylopectin. Fingerprint B-chains are probably entirely included within the building blocks, while longer B-chains of the short chain category are suggested to be involved to a large extent in the interconnection of two, or maybe three, building blocks and to a large part be found as branches to the backbone (Bertoft et al., 2012).

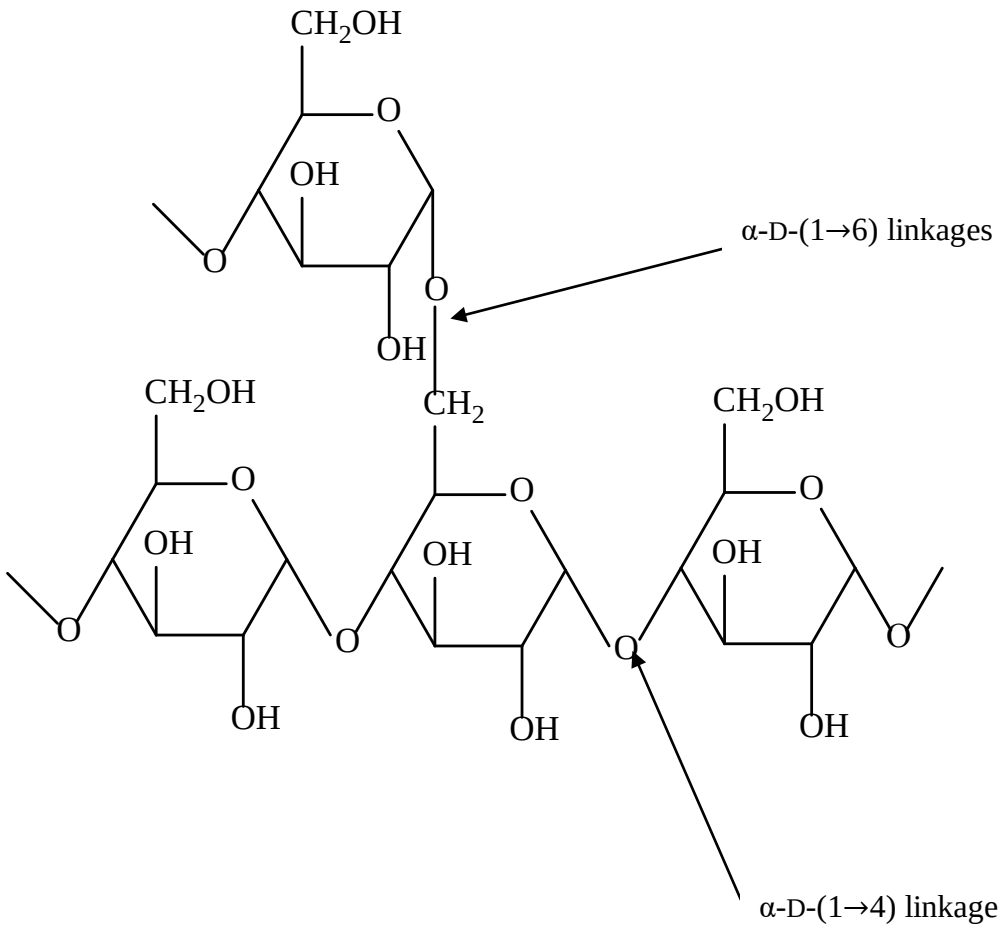



**Figure 2.5** Schematic diagram of amylopectin

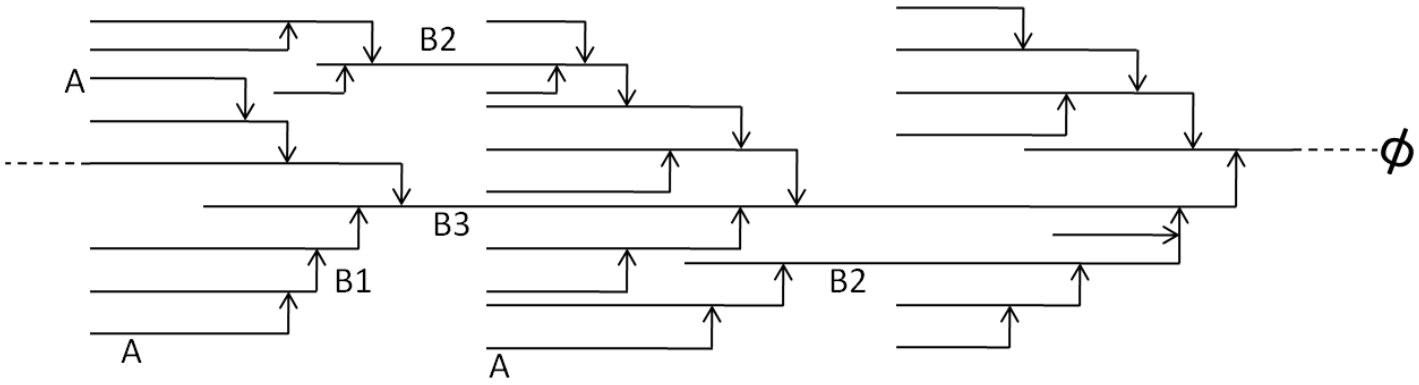



**Figure 2.6** Cluster model of amylopectin indicating A, B1-B3 chains. ϕ is the reducing chain-end (Adapted from Hizukuri, 1986)

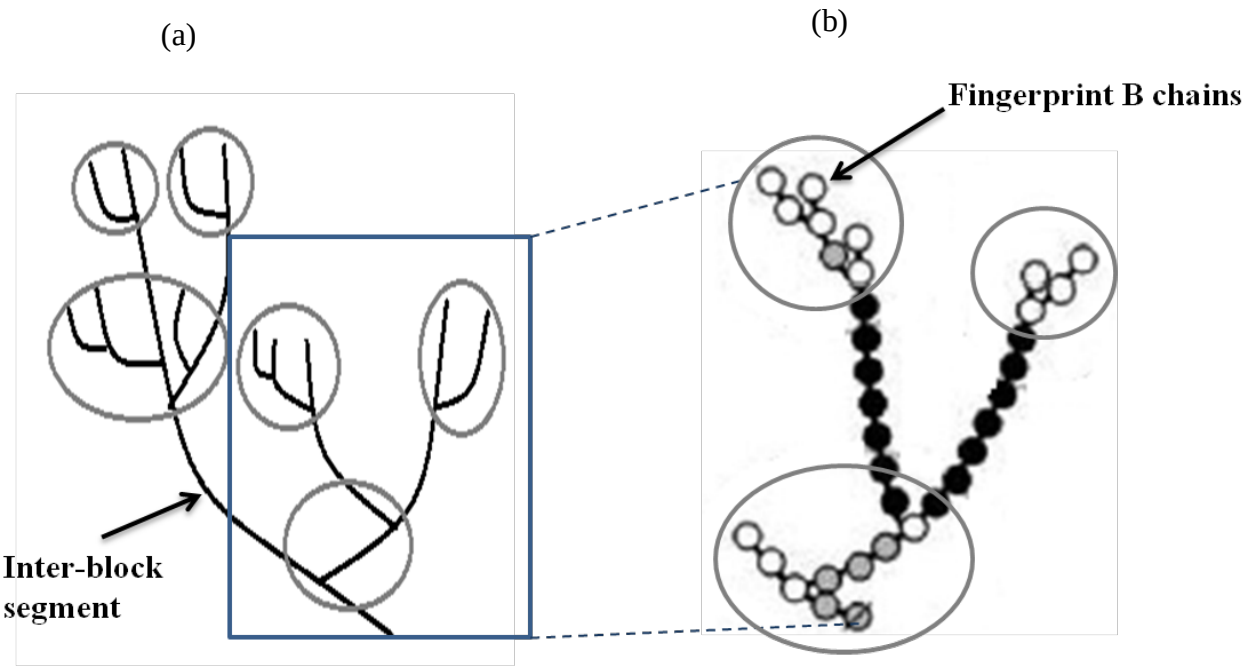



**Figure 2.7** Schematic representation of a building block backbone model of amylopectin: (a) building blocks are encircled (b) an enlargement of a part of the cluster. Circles indicate glucosyl residues (Adapted from Pérez & Bertoft, 2010).

### 2.6.1.3 Starch crystallinity

Starch is biosynthesized as semi-crystalline granules with varying polymorphic types and degrees of crystallinity (Buléon et al., 1998). Wide angle X-ray scattering (WAXS) is used to identify crystal structure(s) and regular molecular arrangements present in native and processed starch (Kavesh & Schultz, 1969). Plant starches are generally classified into three types (A, B, and C) according to the type of X-ray diffraction pattern. A-type pattern can be found typically in cereals, B type in potato, high amylose maize, and barley starches, and C types were reported in pulse starches (Buléon et al., 1997; Frost, Kaminski, Kirwan, Lascaris, & Shanks, 2009). The differences between A and B starch are resulted from water content and the manner in which double helices are packed in the respective crystals (Imberty, Buléon, Tran, & Péréz, 1991). The A-type starch has a monoclinic unit cell, closely packed, and has low water content. In contrast, the B-type polymorphic starch has a hexagonal unit cell, which is relatively loosely packed with an open channel of water in the unit cell (Fig 2.8) (Imberty, Chanzy, Pérez, Bulèon, & Tran, 1988; Imberty et al., 1991). Sarko and Wu (1978) studied the crystal structure of A-, B-, and C-polymorphs of amylose by the combination of X-ray diffraction and computer-based structure refinement, and concluded that C-type structure is a mixture of A and B unit cells and therefore intermediate between A and B forms in packing density. Another polymorph often found starch is the V-type, which arises from single amylose helices that are complexed with lipids (Morrison, Law, & Snape,1993; Lopez-Rubio, Flanagan, Gilbert, & Gidley, 2008).

Hydration of starch granules leads to increase starch crystallinity but does not change the crystal type (Cheetham & Tao, 1998). Starch crystallinity has been shown to be influenced by amylopectin content, average amylopectin chain length, orientation of the double helices (within the crystallites) to the X-ray beam, crystallite size, and starch moisture content (Gunaratne &

Hoover, 2002; Jayakody, Hoover, Liu, & Weber, 2005). Cheetham and Tao (1998) showed that the crystal type of maize starches changes from A to B via C with an increase in amylose content, resulting a decrease in degree of crystallinity from 41.8% to 17.2% across a range of apparent amylose content from 0% to 84%. Similarly, crystallinity decreased with increasing amylose content in wheat (Lan et al., 2008) and barley (Waduge, Hoover, Vasanthan, Gao, & Li, 2006) starches.

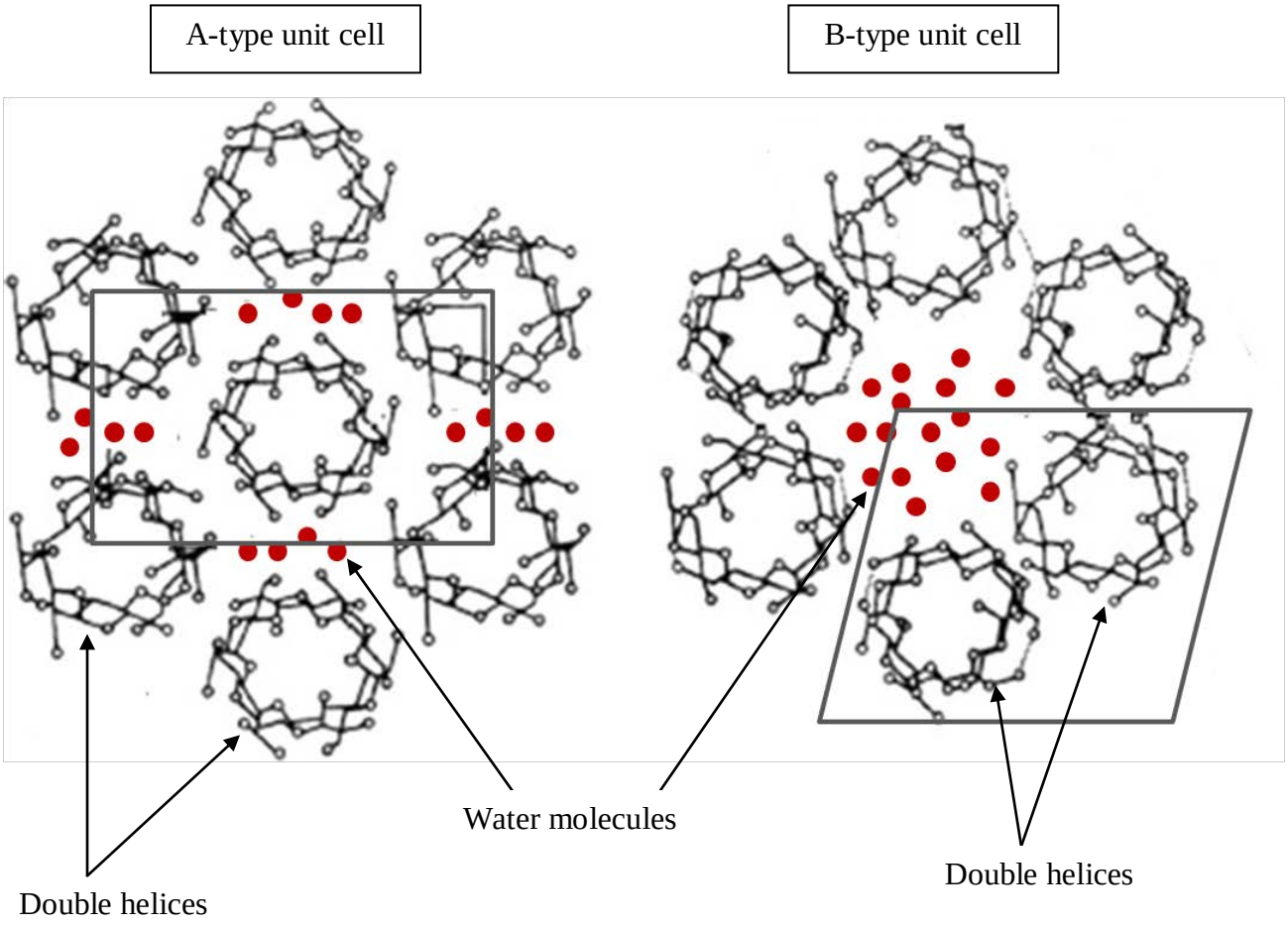



**Figure 2.8** Double helical arrangement of A-type and B-type crystallites in starch (Adapted from Wu & Sarko, 1978a;1978b)

## 2.7 Minor components of starch

Beside amylose and amylopectin, starch granules also comprise minute amounts of proteins, lipids, and phosphorus. Granule-bound proteins were found at both the surface of granules and in interior parts (Pérez & Bertoft, 2010). Granule associated proteins may be associated with lipids on granule surface (Baldwin, 2001). Purified starches contain <0.6% protein (Tester et al., 2004). Integral proteins (~50–150 kDa) have a higher molecular weight than surface proteins (~15–30 kDa) and include residues of enzymes involved in starch synthesis, especially starch synthase (Baldwin, 2001).

Lipids are found in low amounts (up to 1.5%) in many starches, especially cereal starches (Morrison, Milligan, & Azudin, 1984). Cereal starches contain integral lipids in the form of lysophospholipids (LPL) and free fatty acids (FFA) which are positively correlated with the amylose fraction and the LPL may account for up to ~2% of starch weight (in high amylose cereal starches) (Tester et al., 2004). The starch granules may acquire surface lipids, which are mainly triglycerides, and to a lesser extent free fatty acids, glycolipids and phospholipids (Copeland et al., 2009). Part of the amylose fraction within lipid-containing granules exists as an amylose-lipid inclusion complex and the presence of these complexes in native starch granules is evident from $^{13}C$ cross polarisation-magic-angle spinning/nuclear magnetic resonance ($^{13}C$ CP-MAS/NMR) (Morrison et al., 1993). The lipid content of native starches is highly correlated with amylose content, as lipid content increases with increasing amylose content (Copeland et al., 2009). The formation of starch-lipid complexes and their impact on the functionality of starch systems are of interest, since amylose-lipid complexes reduces the solubility and swelling power, alters the rheological properties of pastes, increases gelatinization temperature, reduces gel rigidity, retards retrogradation, and reduces the susceptibility to enzyme hydrolysis (Crowe,

Seligman, & Copeland, 2000; Ozcan & Jackson, 2002; Copeland et al., 2009; Chao, Yu, Wang, Copeland, & Wang, 2017).

Starches also contain relatively minute quantities (<0.4%) of minerals which include calcium, magnesium, phosphorus, potassium, and sodium. Most of the minerals do not have a functional significance except for phosphorus (Tester et al., 2004). The level of starch phosphorylation varies considerably with the botanical origin of the starch.In root and tuber starches, phosphorous (0.01 – 0.1%) is covalently linked to the starch as phosphate monoesters, whereas in cereal starches (0.02 – 0.06%) it occurs mostly as contaminating phospholipids (Lim & Seib, 1993; Blennow, Nielsen, Baunsgaard, Mikkelsen, & Engelsen, 2002; Gunaratne & Hoover, 2002). Phosphate groups are monoesterified to amylopectin molecule in the positions of C-6 or C-3 of the glucosyl unit (Lim, Kasemsuwan, & Jane, 1994). The amount of phosphate monoesters in starch strongly influences its physicochemical properties by increasing the hydration capacity of starch pastes after gelatinization and, asa result, the starch-phosphate content is correlated to starch-paste peak viscosity and gel-forming capacity (Wiesenborn, Orr, Casper, & Tacke, 1994; Viksø-Nielsen et al., 2001). Starch phosphorylation constitutes an integral part of the starch-biosynthetic pathway (Blennow et al., 2002). A protein (designated as R1) that is bound to potato starch granule has been identified and circumstantial evidence suggests that it is involved in the phosphorylation of starch (Lorberth, Ritte, Willmitzer, &Kossmann, 1998),and this protein wasrecently identified as an α-glucan water dikinase(GWD) (Ritte et al., 2002). Phosphate groups will align with or project from the helix surface (Fig. 2.9), which might affect the stability and packing of the helices and hence the crystallinity of the starch (Belennow et al., 2002).

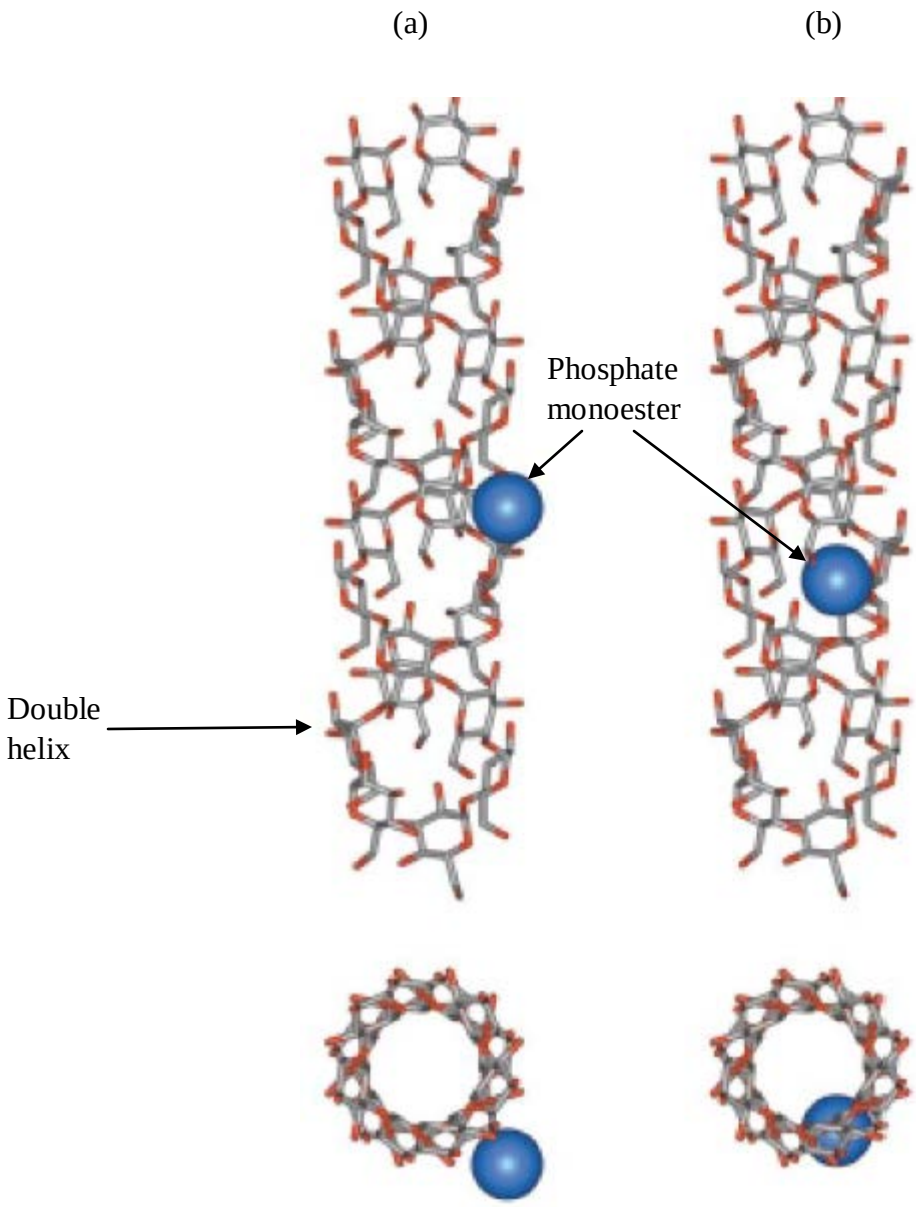



**Figure 2.9** A molecular model of phosphorylated starch (crystalline domain). The helices are phosphorylated on the same glucose residue, at the C-3 (a) andC-6 (b) positions (Adapted from Blennow et al., 2002).

## 2.8 Starch properties

### 2.8.1 Granule swelling and amylose leaching

Starch granules swell when heated in the presence of water, which leads to loss of ordered structures within the native granule (Debet & Gidley, 2006). Swelling curves of waxy and non-waxy cereal starches, at various temperatures up to 85$^{o}$C, were characterized by an initial phase of slight swelling, a second phase of rapid swelling, and a final stage of maximum swelling (Tester & Morrison, 1990a). Tester and Morrison (1990a) have shown, by comparing waxy and non-waxy starches, that granule swelling of starch is primarily a property of amylopectin. Crystallites within the amylopectin molecules determine the onset of swelling and gelatinization (Tester & Morrison, 1990a, b). High proportion of long chains (DP $\geq$ 37) in amylopectin in wheat starches increased granule swelling (Sasaki & Matsuki, 1998). However, in potato and cassava starches, longer chains (DP > 25) inhibited swelling, whereas short chains (DP 6–14) stimulated swelling (Gomand, Lamberts, Visser, & Delcour, 2010).

The presence and amount of non-carbohydrate constituents (proteins/lipids) are important in granule swelling. Debet and Gidley (2006) have identified three types of starch swelling behaviour: starches that swell rapidly at temperatures above that required for thermal gelatinization (e.g. tapioca, waxy maize, potato), starches that have restricted swelling above gelatinization temperature that is related to surface lipids/proteins (e.g. wheat, maize), and starches that have highly restricted swelling not related to surface lipids/proteins (e.g. high amylose maize and potato). In normal cereal starches, amylose and lipids actively inhibit swelling, except in barley starch above 60$^{o}$C where they only act as diluents (Tester & Morrison, 1990a). Inhibition of granule swelling by amylose can be attributed to the interaction between starch chains (amylose-amylose and/or between amylose and outer branches of amylopectin)

(Vamadevan & Bertoft, 2015). Swelling is favoured by phosphate ester groups (Galliard & Bowler, 1987). Phosphate monoesters have the properties of an anionic polyelectrolyte when they are dispersed into aqueous solutions and then repel one another facilitating granule swelling (Vamadevan & Bertoft, 2015).

Two different methods have been used to study granule swelling. Swelling power is the earliest method which measures the ratio of the wet weight of the sedimented gel to its dry weight (Leach, McCowen, & Schoch, 1959). However, this method cannot distinguish intragranular water from intergranular water. The swelling factor method (Tester & Morrison, 1990a), that uses blue dextran dye which dissolves in supernatant and interstitial water but not in the intragranular water, gives the ratio of the volume of swollen granules to the initial volume, and have been widely used to measure the degree of swelling.

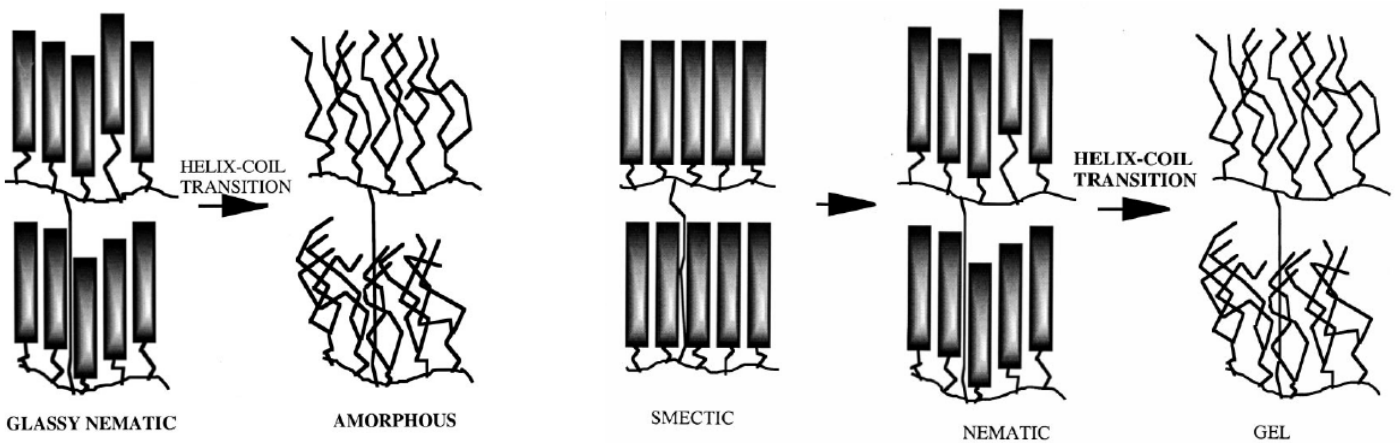
Granular swelling is also accompanied by leaching of starch molecules from the granules.Tester and Morrison (1990a) showed in waxy and non-waxy wheat, corn, and barley starches, that leaching of polysaccharide (amylose and/or amylopectin, depending on the starch) is highly correlated with swelling factor. Amylose of mainly low molecular weight diffuses out at low temperatures, whereas larger molecules leach at higher temperatures (Vamadevan & Bertoft, 2015). The extent of amylose leaching has been shown to influence by: 1) the extent of the interaction between glucan chains (amylose-amylose and/or amylose-amylopectin) (Lan et al., 2008; Gomand et al., 2010), 2) the amount of lipid-complexed amylose chains (Hoover & Vasanthan, 1994a; Gunaratne & Hoover, 2002)., 3) the phosphate content (Hoover, 2010), and 4) the granule size (Lindeboom, Chang & Tylera, 2004).

### 2.8.2 Gelatinization

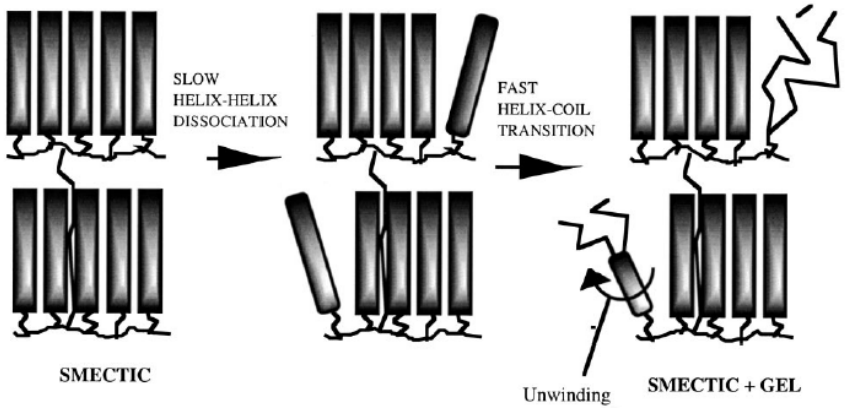
When starch granules are heated in excess water, they undergo an irreversible phase transition, referred to as gelatinization, in which the highly ordered structure is disrupted (Wang & Copeland, 2013). Collapse of crystalline order (breaking of hydrogen bonds) within the starch granules manifests itself as irreversible changes in properties, such as water uptake, granule swelling, pasting, loss of birefringence (radial orientation of starch crystallites), loss of crystalline order, unwinding and dissociation of the double helices, and starch solubility (Atwell, Hood, Lineback, Varriano-Martson, & Zohel, 1988; Hoover, 2001; Biliaderis, 2009). During the first stage of gelatinization, the granules swell as hydrogen bonds in amorphous regions are disrupted and water, which acts as a plasticizer, is absorbed. As the temperature increases, hydration and swelling expand and transmit disruptive forces into the crystalline regions (Cooke & Gidley, 1992; BeMiller, 2011). Ratnayake and Jackson (2007) have described starch phase transition as a three-stage process in which the starch granules absorb water increasing the starch polymer mobility in amorphous regions, formation of new intermolecular interactions and an increase in hydrothermal effects take place causing the disruption of overall granule structure. Gelatinization mainly depends on starch/water ratio, but also on different processing parameters such as temperature and heating rate (Sakonidou, Karapantsios, & Raphaelides, 2003)

Mechanism of granule disassembly during starch gelatinization was explained by liquid-crystalline model for gelatinization (Fig 2.10) in which it was assumed that hydrated amylopectin has a similar structure to that of a branched side-chain liquid-crystalline polymer of a smectic (a state of a liquid crystal in which the molecules are oriented in parallel and arranged in well-defined planes) or nematic (a state of a liquid crystal in which the molecules are oriented in parallel but not arranged in well-defined planes) type (Waigh, Gidley, Komanshek, & Donald,

2000). In the nematic state, helices are not aligned into lamellae, whereas in the smectic state, the mesogens (rigid units corresponding to double helices) are aligned (Waigh et al., 2000). At low water level (<5 w/w%), a direct transition from the glassy nematic/isotropic to the amorphous state occurs at elevated temperature and the single peak corresponds to a helix-coil transition, whereas at intermediate water level (5-40 w/w%), biphasic peak results in which the first endotherm is associated with the smectic–nematic/isotropic transition and the second endotherm corresponding to the helix–coil transition. At excess water conditions (>40 w/w %), the ultimate loss of the smectic crystalsis assumed to occur simultaneously with helix dissociation (Waigh et al., 2000).




(a) (b)




(c)

**Figure 2.10** The molecular disassembly of starch granules during gelatinization: a) The single stage process in the gelatinization of starch at low water contents. b) The two stage process involved in the gelatinization of starch in limiting (intermediate) water. c) The two stage process involved in the gelatinization of starch in excess water. The first stage involves a slow dissociation of the helices side by side. Immediately a helix-coil transition occurs as a secondary effect (Waigh et al., 2000, Copyright Elsevier, reproduced with permission)

A number of techniques, including differential scanning calorimetry (DSC), thermo-mechanical analysis (TMA), wide angle X-ray scattering (WAXS), small angle X-ray scattering (SAXS), small angle neutron scattering (SANS), Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance spectroscopy (NMR), light scattering, and optical microscopy have been used to monitor starch gelatinization (Jenkins & Donald, 1995; Ratnayake & Jackson, 2007; Biliaderis, 2009; Wang & Copeland, 2013; Vamadevan & Bertoft, 2014). DSC, which measures the heat released or absorbed by a material during phase transitions, is widely used to determine gelatinization temperature and energy absorbed by the starch–water systems during gelatinization (Stevens & Elton, 1971). DSC measures gelatinization transition temperatures (onset [To], melting [Tm], and conclusion [Tc]), and the enthalpy (ΔH) of gelatinization (Vamadevan & Bertoft, 2014).

The extent of crystalline perfectionis reflected in the gelatinization temperatures (Tester, 1997), and ΔH represents the number of double helices that unravel and melt during gelatinization (Cooke & Gidley, 1992). Starches from different botanical sources, varying in composition, showed different transition temperatures and enthalpies of gelatinization and these differences may be attributed to the differences in the degree of crystallinity (Singh, Singh, Kaur, Sodhi, & Gill, 2003). Generally, high transition temperatures and a narrow endothermic peak indicate a higher molecular order or more stable crystals (Vamadevan, Bertoft, & Seetharaman, 2013a). Fredriksson, Silverio, Andersson, Eliasson, and Åman, (1998) studied gelatinization in waxy and non waxy starches and showed that the amylose content was negatively correlated to the onset and the peak temperatures of gelatinization. Since amylopectin plays a major role in starch granule crystallinity, the presence of amylose lowers the melting point of crystalline regions and the enthalpy of gelatinization (Flipse, Keetels, Jacobson, & Visser, 1996). The differences in

gelatinization temperatures in different starches can be attributed to molecular structure of amylopectin (unit chain length, extent of branching), starch composition (amylose to amylopectin ratio, amount of lipid complexed amylose chains, phosphorous content), and granular architecture (crystalline to amorphous ratio) (Gunaratne & Hoover, 2002).

Starch gelatinization has important role in food processing, since most starch consumed by humans has undergone some form of processing or cooking, which causes native starch granules to gelatinize (Wang & Copeland, 2013). Gelatinized starches are used in foods as a thickening in sauces, puddings, cream and other food products. The gelatinization of starch also influences the characteristics and quality of food, elasticity and softness of paste products, digestibility and palatability (Chiang & Johnson, 1977).

### 2.8.3 Retrogradation

When starch granules are heated in excess water above their gelatinization temperature, irreversible swelling occurs followed by amylose leaching into the solution (Hoover, 2001). Upon cooling, the starch chains (amylose and amylopectin) in the gelatinized paste interact, forming more ordered structure (Fig. 2.11). These molecular interactions that occur after cooling are known as retrogradation (Hoover et al., 2010). The formation of an amylose matrix gel is attributed to the initial gel firmness during retrogradation, followed by slow increase in gel firmness due to reversible crystallization of amylopectin (Ring et al., 1987). The long-term development of gel structure and crystallinity of processed starch, which are involved in the staling of bread and cakes, are considered to be due to retrogradation of amylopectin (Tran, Okadome, Murata, Homma, & Ohtsubo, 2001; Gray & BeMiller 2003). During retrogradation, amylose forms double helical associations of 40–70 glucose units (Jane & Robyt, 1984) whereas

amylopectin crystallization occurs by association of the outermost short branches (Ring et al., 1987).

Starch retrogradation is a non-equilibrium thermo reversible recrystallization process, which takes place in three consecutive steps: nucleation (formation of crystal nuclei), propagation (crystal growth from the nuclei formed during nucleation), and maturation (Silverio, Fredriksson, Andersson, Eliasson, & Åman, 2000). The rate of retrogradation is dependent on the concentration of the paste, the molecular size, the pH and the temperature (Slade & Levine, 1988). The storage temperature has been shown to influence the rate of both nucleation and propagation (Slade & Levine, 1987). Retrogradation is accompanied by a series of physical changes such as increase in crystallinity,viscosity and turbidity of pastes, gel firmness, exudation of water (syneresis), gel network formation and appearance of a "B" type X-ray pattern (Hoover, 1995; Hoover et al.,2010). For non-waxy starches, retrogradation results in the transformation of a starch paste into a firm gel consisting of a 3-dimensional network whereas, waxy starch pastes on retrogradation form a soft gel, which contains aggregates but no network (Tang & Copeland, 2007).

The variation in thermal properties of starches after gelatinization and during refrigerated storage may be attributed to the variation in amylose to amylopectin ratio, size and shape of the granules (Singh et al., 2003), and presence/absence of lipids (Perera & Hoover, 1999; Singh et al., 2003), proteins (Appelqvist & Debet, 1997), and phosphorous (Thygesen, Blennow, & Engelsen, 2003). Starch retrogradation has been shown to be influenced by amylopectin chain length distribution (Lu, Chen, & Lii, 1997). Shi and Seib (1992) showed that the retogradation of waxy maize, barley, and rice starches was directly proportional to the mole fraction of branches with DP 14-24, and inversely proportional to the mole fraction of branches with DP 6-9.

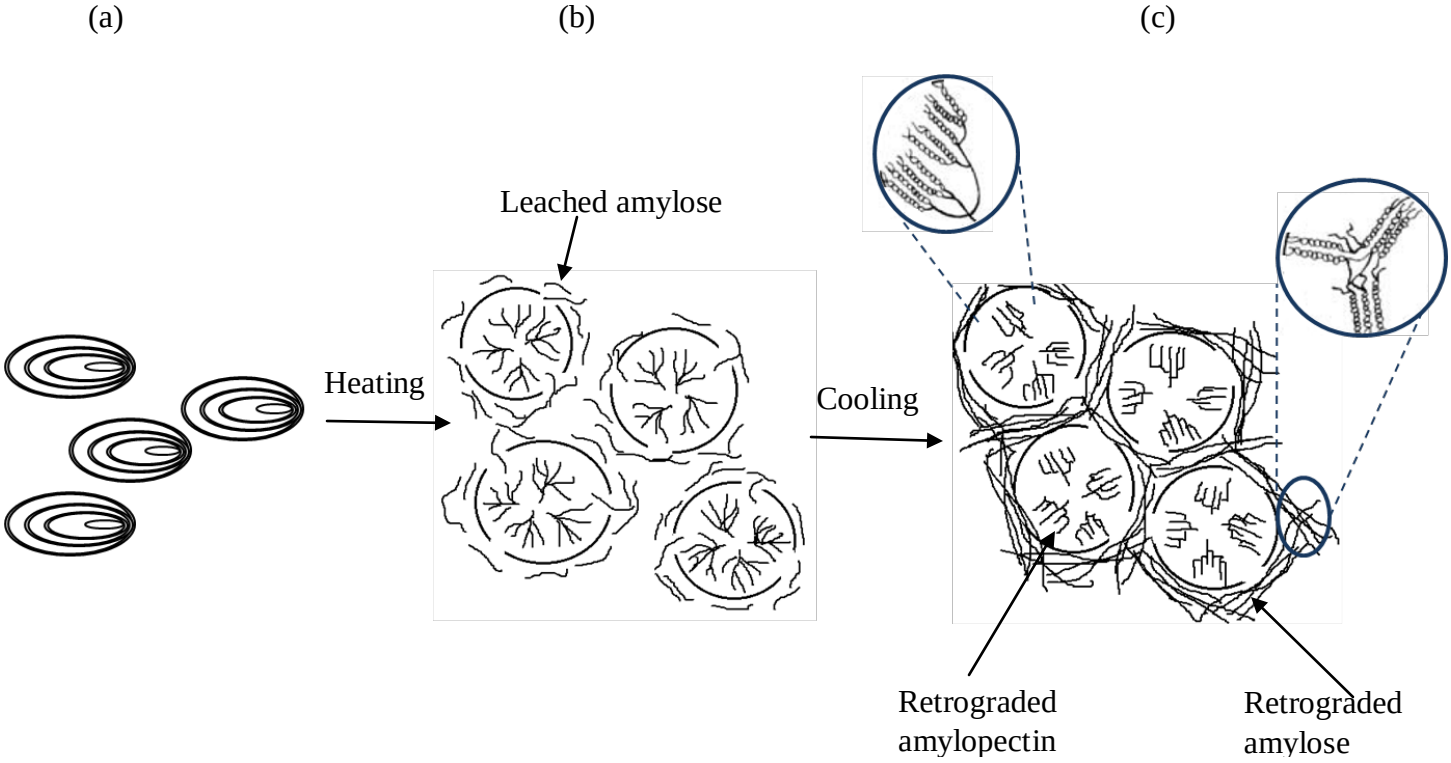



**Figure 2.11** A schematic representation of the processes and structures observed during heating and storage of aqueous suspensions of granular starch: (a) native starch granules, (b) gelatinization, associated with swelling and amylose leaching, (c) retrogradation during cooling (Adapted from Goesaert et al., 2005).

Since starch retrogradation is a complex process involving a series of molecular and physicochemical events, a variety of physical and chemical methods have been applied to investigate the changes that take place in starch properties (Wang, Li, Copeland, Niu, & Wang, 2015). These changes can be monitored with the use of WAXS, $^{13}$C-NMR, DSC, FTIR, spectrophotometry and microscopic imaging (Hoover, 2001; Ambigaipalan, Hoover, Donner, & Liu, 2013; Wang et al., 2015). DSC has proven to be an extremely valuable and sensitive tool to characterize starch retrogradation, although no single method can give a complete picture of retrogradation properties at both macroscopic and molecular levels (Karim, Norziah, & Seow, 2000).

Starch retrogradation is often considered to be undesirable because it is responsible for the staling of bread and other starch-rich foods, resulting in reduced shelf-life and consumer acceptance, however, it is found to be useful in some food applications such as production of breakfast cereals, parboiled rice, in the manufacture of croutons and bread crumbs, due to modification of the structural, mechanical, and sensory properties (Karim et al., 2000; Ottenhof & Farhat, 2004).

### 2.8.4 Acid hydrolysis

Acid-hydrolyzed starches are normally prepared by exposing starch granules to dilute aqueous or alcoholic solutions of mineral acid (hydrochloric or sulfuric acid) below the gelatinization temperature for various time periods (Wang & Copeland, 2015). The ratio of the cold to hot paste viscosity and the required gel texture influence the parameters used for acid hydrolysis, hence, when the desired viscosity or fluidity is attained, the resultant starch is recovered by neutralizing, washing, centrifugation, and drying (Hoover, 2000). Acid hydrolysis alters the structure of starch granules resulting changes in the functional properties. The glycosidic bond between monosaccharide units in a polysaccharide is cleaved in the presence of acid and heat. Initially, an electrophilic attack is carried out by the hydroxonium ion ($H_3O^+$) on the oxygen atom of the α-D-(1→4) glycosidic bond (Fig. 2.12a) followed by the moving of the electrons in one of the carbon–oxygen bonds onto the oxygen atom (Fig. 2.12b) to generate a carbocation intermediate (Fig. 2.12c). Lastly, the carbocation intermediate reacts with water (Fig. 2.13d) to regenerate a hydroxyl group (Fig. 2.12e) (Hoover, 2000).

Acid hydrolysis shows a two-stage hydrolysis pattern: a fast-initial rate followed by a slower subsequent rate (Robin, Mercier, Charbonniere, & Guilbot, 1974; Jane, Wong, & McPherson, 1997; Hoover, 2000; Jayakody & Hoover, 2002). The fast-initial rate is attributed to the hydrolysis of the amorphous regions within starch granules, whereas the later slow rate is attributed to the hydrolysis of crystalline regions (Jayakody & Hoover, 2002). The slower hydrolysis rate of the crystalline domains has been attributed to the dense packing of starch chains within the crystallites which prevent rapid penetration of $H_3O^+$ and very slow occurring of the chair to half chair transformation (required for hydrolysis of the glycosidic bond) due to immobilization of the sugar conformation within the starch crystallites (Hoover, 2000).

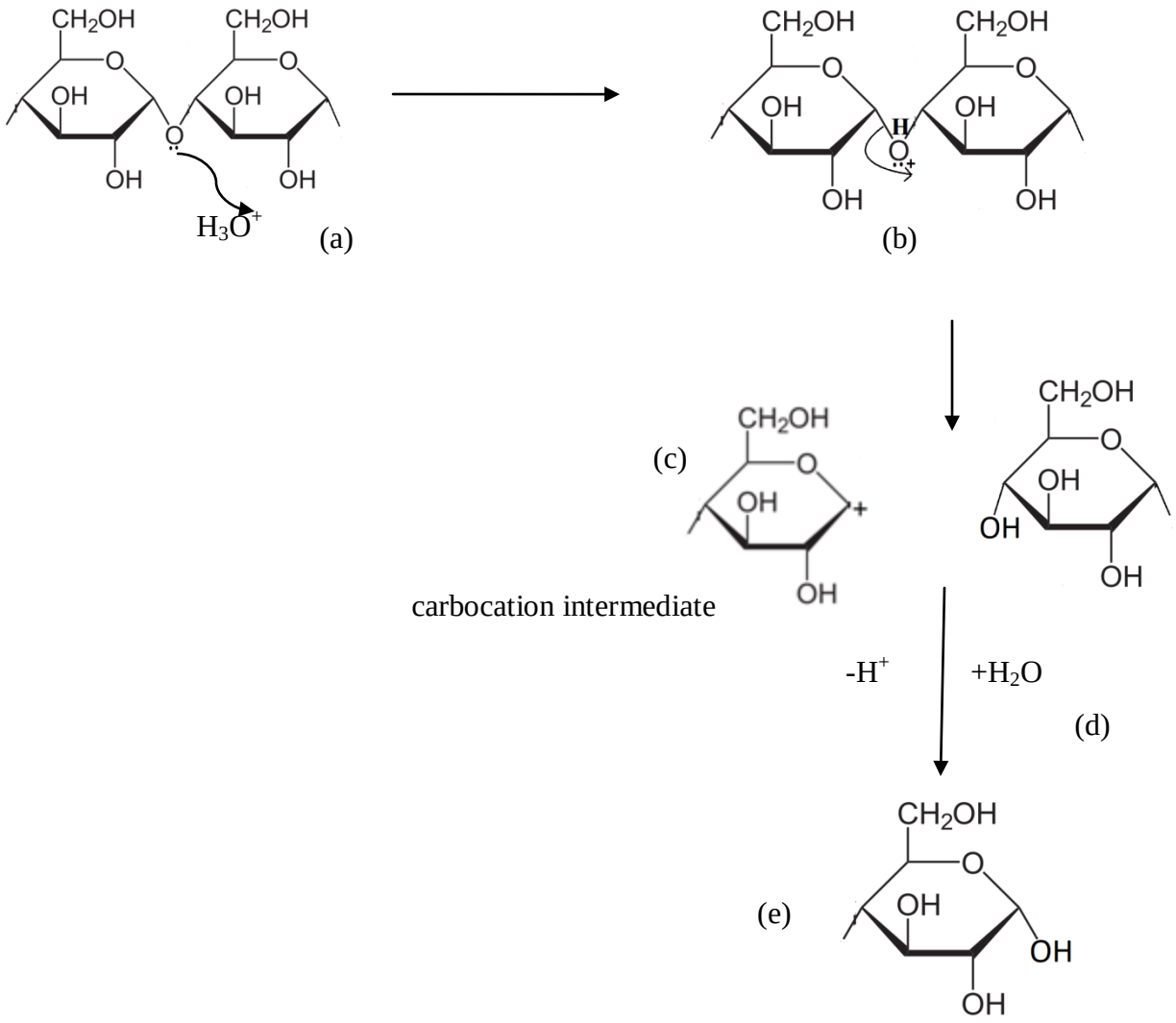



**Figure 2.12** Mechanism of acid hydrolysis: (a) electrophilic attack of $H_3O^+$ on the oxygen atom of the α-D-(1→4) glycosidic linkage, (b) electrons in one of the C-O bonds moves to the oxygen atom, (c) generation of unstable, high-energy carbocation intermediate, (d) reaction of carbocation intermediate with water, (e) regeneration of OH group (Adapted from Hoover, 2000)

The first stage of hydrolysis is influenced by the granule size, pores on the surface, amylose content, and the amount of lipid-complexed amylose chains, whereas the second step of hydrolysis affected by the amylopectin content, the distribution of α-D-(1→6) branches between the amorphous and crystalline lamellae, and degree of the double helical packing within the crystallites (Le Corre, Bras, & Dufresne, 2010; Wang & Copeland, 2015). High amylose starches solubilized slowly during acid treatment in comparison to low amylose starches (Vasanthan & Bhatty, 1996; Jane et al., 1997; Jayakody & Hoover, 2002; Nakazawa & Wang, 2003). The lower susceptibility of high amylose starches to acid hydrolysis is attributed to either the greater extent of starch chain interactions within the amorphous regions (Hoover & Manuel, 1996) or slower penetration of acid into the granules due to the restricted swelling of high amylose starches (Nakazawa & Wang, 2003).

### 2.8.5 Enzyme hydrolysis

Starch is the main carbohydrate in human nutrition and the digestion of starch is not a simple and single process. The major physiological properties of starch are the release of glucose as a source of energy for the body and the timeline of digestion (Lehman & Robin, 2007). There are a number of different structural scales to be considered in starch-based foods, ranging from the distribution of individual branching structures of macromolecules of starch granules to the macroscopic structure of the grain which contains not only the starch but also proteins, lipids and non-starch polysaccharides (Dona et al., 2010).

The enzymatic digestion of starch often involves a combination of one or more endo-enzymes and exo-enzymes that convert the products of endo-enzymes into monomer units or act directly (Zhang, Dhital & Gideley, 2013). The hydrolysis pattern of starch granule is commonly studied using α-amylase and glucoamylase. The α-amylase (EC 3.2.1.1) is a well-known endo-amylase

which cleaves α-D-(1→4) glycosidic bonds of amylose and amylopectin leading to the formation of oligosaccharides with varying length with an α-configuration and α-limit dextrins, which constitute branched oligosaccharides (Van Der Maarel, Van der Veen, Uitdehaag, Leemhuis, & Dijkhuizen, 2002). Glucoamylase (EC 3.2.1.3) is an exo-acting enzyme which hydrolyzes both α-D-(1→4) and α-D-(1→6) linkages of starch polymers from their non-reducing ends producing β glucose (Zhang et al., 2013). Apart from these, there are other enzymes such as, β-amylase (EC 3.2.1.2), an exo-acting enzyme which hydrolyzes α-D-(1→4) linkages and produce maltose as a major end product and β-limit dextrin. Isoamylase (EC3.2.1.68) and pullanase type I (EC 3.2.1.41) are debranching enzymes that hydrolyze only α-D-(1→6) linkage (Van Der Maarel et al., 2002).

Enzyme hydrolysis of granular starch is a heterogeneous reaction, which involves a reaction between an enzyme in a solution and a solid substrate represented by the granules (Tahir, Ellis, Bogracheva, Meares-Taylor, & Butterworth, 2010). The steps involved in enzyme digestion include the diffusion of enzymes to the granule surface, followed by adsorption and subsequent catalytic events (Leloup, Colonna, & Ring, 1990; Uthumporn, Zaidul, & Karim, 2010). Many different modes of enzyme attack have been identified in various starch sources such as pin-hole, medium-sized holes, single holes in individual granules, sponge-like erosion, and surface erosion (Sujka & Jamroz, 2007). Enzymes can erode the complete granule surface (exo-corrosion) or digest channels at precise locations on the surface towards the center (endo-corrosion), depending on the enzyme and variety of starch (Dona et al., 2010).

Native starch is digested slowly compared to processed starch as processing conditions disrupt granule integrity and crystallinity, thereby increasing the accessibility of glucans to enzymes (Blazek & Gilbert, 2010). Starch hydrolysis by α-amylase depends on several features such as

granule size, crystallinity, extent of disruption of amylopectin clusters, and lipid complexed amylose chains (Lan et al., 2008). Smaller granules hydrolyse to a greater extent than the larger granules due to relatively larger surface area per unit mass in smaller granules, which may increase the extent of enzyme binding and ultimately result in greater hydrolysis (Naguleswaran, Vasanthan, Hoover, & Bressler, 2013). The surface characteristics such as presence of pores, channels, and cavities have been reported to influence the rate of enzyme hydrolysis by increasing the surface available for the enzymatic reactions. Pores provide access for initial enzyme attack that causes the enzyme to penetrate to the granule interior (Sujka & Jamroz, 2007). Amylose-lipid complex formation affects enzyme susceptibility by reducing starch granule swelling and thereby reducing the enzyme accessibility to granule interior (Cui & Oates, 1999) and further, lipid complexed amylose is more resistant to digestive enzymes than amylose (Hoover & Manuel, 1996; Cui & Oates, 1999).

The crystallite structure and the packing of the amorphous phase influence the enzymatic susceptibility (Colonna, Leloup, & Buleon, 1992). The arrangement in A-type or B-type crystallites markedly influences digestibility and a higher susceptibility of A-type crystallites to enzyme hydrolysis compared to B-type crystallites has been reported (Jane et al., 1997; Lehman & Robin, 2007). Jane et al. (1997) have shown that A-type starches have more short chains (DP 6-12) and branch points which are scattered in crystalline regions, creating weak points, which are more susceptible to enzymatic hydrolysis. Generally, tuber starches are more resistant to enzymatic hydrolysis than cereal starches, due to a higher granule surface, their surface properties, presence of porous structures in cereal starches and the supramolecular arrangement (Lehman & Robin, 2007).

### 2.8.6 Starch nutrition fractions

Starch and starch containing foods can be classified according to their digestibility, which is generally characterized by the rate and the duration of the glycemic response (Singh, Dartois & Kaur, 2010). According to *in vitro* measurement of starch digestibility, starch can be divided into three groups, namely rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) (Englyst, Englyst, Hudson, Cole, & Cummings, 1999). RDS is the fraction of starch granule that causes a rapid increase in blood glucose concentration after ingestion of starch, whereas SDS is the fraction of starch that is digested slowly but completely in the human small intestine (Dona et al., 2010). RS is the sum of the starch and products of starch degradation not absorbed in the small intestine of healthy individuals (Englyst et al., 1999).

RDS is mainly comprised of amorphous and dispersed starch and is found in large amounts in starchy foods cooked by moist heat, such as bread and potatoes (Sajilata, Singhal, & Kulkarni, 2006). RDS content of cereal starches (2.5-90%) is higher than tuber (0.1-21.0%) and legume (3.0- 21.0%) starches. The shortest A chains (DP 5-10) cannot form double helices, and they may disrupt the ordered crystalline structure leading to crystalline defects. Therefore, starches with high amount of shortest A chains are likely to be easily attacked by enzymes leading to higher amount of RDS (Zhang, Venkatachalam & Hamaker, 2006b).

SDS consists of physically inaccessible amorphous starch and raw starch with A- and C- type crystalline structures, while B-type starch is resistant to enzyme hydrolysis (Lee & Moon, 2015). SDS has physical structures that decrease enzyme accessibility and chemical structures that limit the rate of enzyme action (Zhang & Hamarker, 2009). Presence of channels in cereal starches enable migration of enzymes into the center of granule and initiate hydrolysis leading to layer-by-layer inside-out pattern where amorphous and crystalline regions are digested simultaneously

and this side-by-side digestion mechanism of native cereal starches cause slow digestion and produce more SDS (>50%) (Zhang, Ao, & Hamaker, 2006a). Cereal starches which had higher short chains (A+B1) showed a higher proportion of SDS compared to potato starch which had less short chains, suggesting that SDS is associated with the fraction of short chains in amylopectin which contributes to the formation of double helices and crystalline lamellae in the crystalline region, and hence, crystalline region is the major determining factor for the slow digestion properties of native cereal starches (Zhange et al., 2006b).

RS is the fraction of starch that cannot be digested in the small intestine, and may be subject to bacterial fermentation in the large intestine to produce short-chain fatty acids and other products (Haralampu, 2000). RS positively influences the functioning of the digestive tract, microbial flora, blood cholesterol level, glycemic index and assists in the control of diabetes. RS has been shown to have lower impact on the sensory properties of food as a dietary fibre source compared with traditional sources of fibre, as whole grains, fruits or bran (Fuentes-Zaragoza, Riquelme-Navarrete, Sánchez-Zapata & Pérez-Álvarez, 2010). RS contents of cereal and pulse starches vary with the amylose and amylopectin contents and their chain lengths (Perera, Meda & Tyler, 2010). RS fractions have extremely broad and diverse range and different types ranging from RS1 to RS5. At present, these are mostly defined according to physical and chemical characteristics (Nugent, 2005).

RS1 is entrapped within whole or partly milled grains or seeds and physically inaccessible (Niba, 2002). RS1 includes starch present in plant cells with undamaged cell walls. RS1 fraction is unavailable for digestive enzymes, since the gastrointestinal track lacks enzymes to digest cell wall components such as cellulose, hemicellulose, lignin etc. (Leszczynski, 2004). RS2 is raw starch granules (such as banana and potato) and high-amylose (high-amylose corn) starches

which are protected from enzyme digestion by the conformation or structure of the starch granule. This compact structure restricts the accessibility of digestive enzymes, and accounts for the resistant nature of RS2 (Fuentes-Zaragoza et al., 2010). RS3 are retrograded starches, which may be formed during cooling of cooked foods (Hernández, Emaldi & Tovar, 2008). Foods which are processed with heat and moisture, therefore, contain some RS3 (Sajilata, et al., 2006). RS3 is thermally stable and this property allows it to be stable in most normal cooking operations, enabling its use as a potential ingredient in a wide variety of conventional foods (Haralampu, 2000). During retrogradation double helices are formed by amylose and/or amylopectin and these double helices aggregate to form crystallites which normally consist of B-type crystalline structure (Sajilata et al., 2006). RS3 is affected by lipid content of the starch since lipid complexed amylose is unable to form double helices and cryatllites, leading to low RS3 content (Leszczynski, 2004). Differences in the food processing conditions, such as moisture content, temperature, duration of heating and subsequent cooling, affect the RS3 content (Perera et al., 2010).

RS4 is formed using chemical modifications, such as substitution, conversion, or cross linking, which can prevent starch digestion by blocking enzyme access and forming linkages other than α-D-(1→4) and α-D-(1→6) (Sajilata et al., 2006; Fuentes-Zaragoza et al., 2010). As a result of chemical modification, different components are incorporated into the starch structure. The presence and spatial changes in the starch chains can hinder the accessibility of glycosidic bonds to the enzyme hydrolysis. Some of these linkages cannot be digested by the enzymes present in the human digestive tract leading to formation of RS (Leszczynski, 2004). Amylose-lipid complex has been proposed as RS5, as a result its resistance to enzyme hydrolysis. The presence of amylose-lipid complex in starch granules increases its resistance to enzyme as a result of its

thermal stability. The enzyme resistance of amylose-lipid complex depends on the molecular structure of the lipid and the crystalline structure of the single helices (Hasjim, Ai, & Jane, 2013).

## 2.9 Modification of starch

### 2.9.1 Annealing

Annealing is a hydrothermal treatment that modifies physicochemical properties of starch without destroying the granular integrity. Annealing of starch granules occurs in excess (>60% w/w) or intermediate (40-55%, w/w) moisture content and held at a temperature above the glass transition temperature ($T_g$) but below the onset temperature of gelatinization ($T_o$) for a certain time period (Jacobs & Delcour 1998; Tester & Debon 2000; Jayakody & Hoover 2008; Zavareze & Dias 2011; Ashogbon & Akintayo, 2014; BeMiller & Huber, 2015). The term $T_g$ describes the temperature at which the rigid glassy amorphous regions transform to a mobile rubbery state when heated in the presence of a plasticizer such as water or glycerol. The ratio of water: starch is critical for $T_g$ because water is a very effective plasticizer of amorphous starch (Tester & Debon, 2000). The aim of annealing is to approach $T_g$, that enables starch chain mobility without triggering the gelatinization.

Annealing causes crystalline perfection in starch granules and facilitates interactions between starch chains (AM-AM, AP-AP, AM-AP) which lead to changes in physicochemical properties. Hydration of starch granule increases the mobility of starch chains, allowing the double helices in the crystalline regions to align into more ordered state causing crystalline perfection (Fig 2.13) (Jayakody & Hoover, 2008; Zavareze & Dias, 2011). Jayakody and Hoover (2008) discussed two models for the mechanism of annealing in semi-crystalline polymers as ‘sliding diffusion’, which is favored by high mobility of starch chains and is the movement of complete molecular

sequence within a crystalline lattice and/or 'complete or partial fusion' of crystals and following re-crystallization of melted starch chains. The extent of crystalline perfection and starch chain interactions occur during annealing varies depending on thestarch source and annealing conditions. Annealing temperature, moisture content, and time vary widely with the botanical origin. Single step annealing has been used in many studies while double and multi-step annealing has been conducted to a limited extent (Jayakody & Hoover, 2008). Different starch: water ratios (1:1, 1:3, 1:4, 1:5) have been used in annealing studies at varying temperatures (40-75$^{o}$C) (Jayakody & Hoover, 2008). Tester, Debon,and Karkalas (1998) have reported that in wheat starch, annealing could be initiated at 20% (w/w) moisture content, but pronounced effects were not obtained unless the moisture content exceeded 60%. Generally, excess moisture content (≥60–65%, w/w) and a temperature 5-15$^{o}$C below onset temperature of gelatinization are the annealing conditions which give significant changes to a given starch (Tester & Debon, 2000; Jayakody & Hoover, 2008). The duration of annealing treatment has varied from minutes to hours to days, engaging a broad range of treatment temperatures, moisture conditions and starch types. In many situations, different starch sources, genotypes, or both exhibited diverse responses to the same annealing conditions (BeMiller & Huber, 2015).

#### 2.9.1.1 Impact of annealing on morphology

Annealing causes several changes to starch structure and properties; however granule morphology is less affected. Many studies reported that granule size or shape was not affected by the annealing treatment (Hoover & Vasanthan, 1993; Waduge et al., 2006; Rocha, Gelizardo, Jane, & Franco, 2012; Wang et al., 2014). However, small deformations in lens-shaped granules of high-amylose and waxy wheat starches were observed (Kiseleva et al., 2005). Slight increase of pore size in waxy barley (Waduge et al., 2006) and an increase in number of pores in waxy

corn (Rocha et al., 2012) upon annealing were observed. Kiseleva et al. (2005) reported that the Maltese-cross and concentric growth rings in wheat starches remain unchanged on annealing, indicating that the granular and lamellar starch structures remain intact.

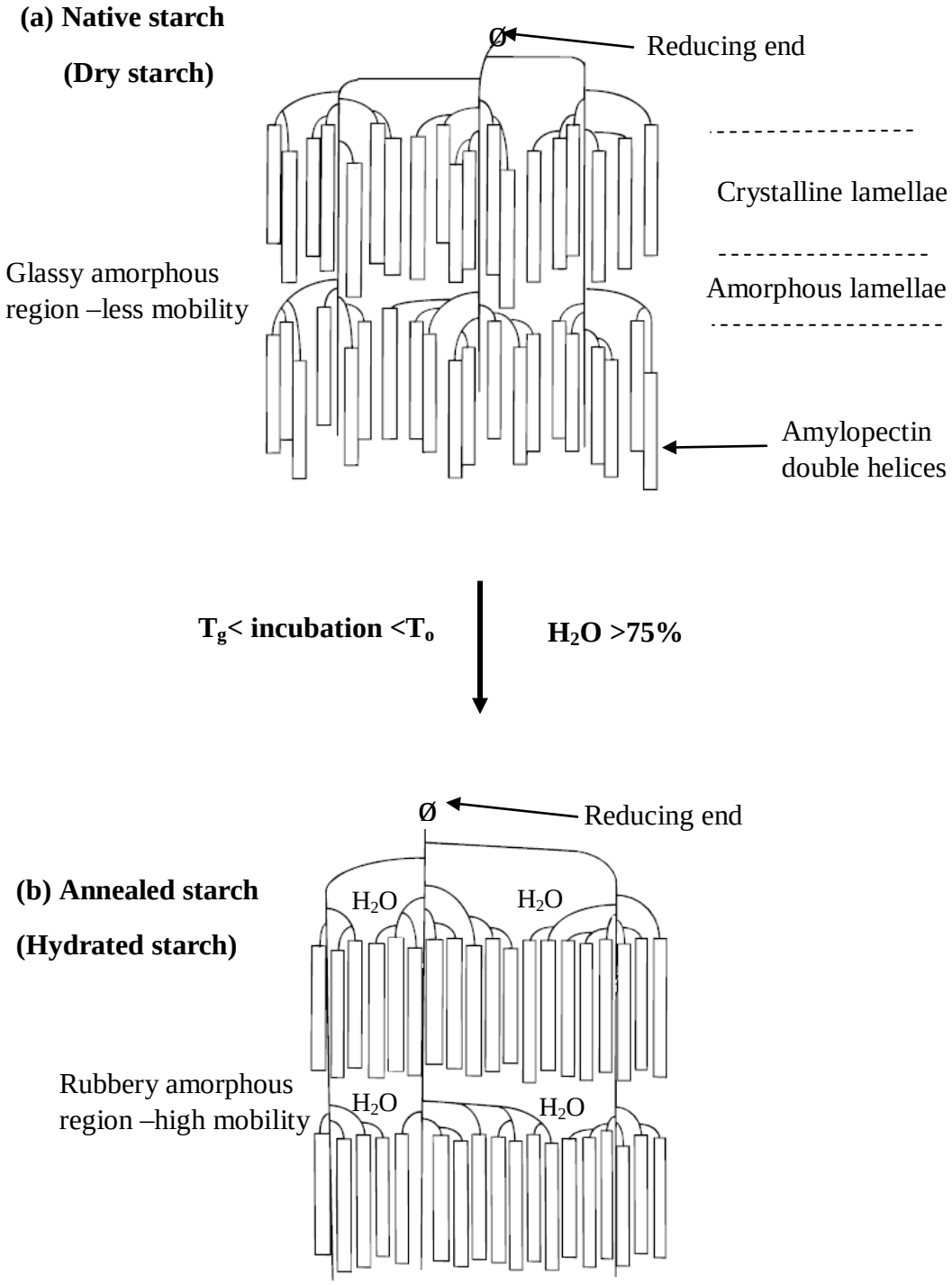



**Figure 2.13** Mechanism of annealing (Adapted from Tester & Debon, 2000)

#### 2.9.1.2 Impact of annealing on starch structure

The major structural changes in annealed starches are as follows: (1) an increase in granular stability (Hoover & Vasanthan, 1993; Jacobs, Eerlingen, Clauwaert & Delcour, 1995; Waduge, et al., 2006; Lan, et al., 2008); (2) crystalline growth and perfection (Hoover & Vasanthan, 1993; Tester, Debon, Davies & Gidley, 1999; Tester, Debon & Sommerville, 2000; Waduge et al., 2006; Lan et al., 2008; Rocha, et al., 2012); (3) an increase in the interactions between starch chains in the amorphous and crystalline regions of the granule (Hoover & Vasanthan, 1993; Tester et al., 2000; Waduge et al., 2006; Lan et al., 2008); (4) lengthening of amylopectin double helices by twisting the unordered ends (Tester et al., 1998,1999; Kiseleva et al., 2004,2005; Vamadevan, Bertoft, Soldatov & Seetharaman, 2013b); (5) an increase in crystallinity (Waduge et al., 2006; Lan et al., 2008; Rocha et al., 2012; Zeng, Ma, Kong, Gao & Yu, 2015). However, annealing has been shown to have no influence on the wide angle X-ray diffraction pattern (Liu, Yu, Simon, Dean, & Chen,2009; Gomand, Lamberts, Gommes, Visser, & Delcour,2012; Rocha, et al., 2012; Wang et al., 2014).

#### 2.9.1.3 Impact of annealing on starch properties

Annealing reduces granule swelling and solubility of starch. The reduction of granule swelling is attributed to the interplay between the extent of crystalline perfection, formation of V-amylose–lipid complexes, and the amylose–amylose and/or amylose–amylopectin interactions (Hoover & Vasanthan, 1993; Waduge et al., 2006; Lan et al., 2008). Annealing strengthen intragranular binding forces (Jacobs et al., 1995) and reduce hydration of the amorphous regions of the starch, decreasing granule swelling, and thereby decreasing solubility (Waduge et al., 2006).

Annealing has been shown to increase gelatinization temperatures (onset [$T_o$], midpoint [$T_p$] and conclusion [$T_c$]), and decrease gelatinization transition temperature range ($T_c$-$T_o$), in all the

starches, regardless of the starch source (Jacobs & Delcour 1998; Tester & Debon 2000; Jayakody & Hoover 2008; Zavareze & Dias 2011; BeMiller & Huber, 2015). The increase in gelatinization temperature has been shown to be most pronounced for $T_o$ and least for $T_c$ (Jayakody & Hoover 2008), since $T_o$ represents melting of weaker crystallites (Nakazawa & Wang, 2003) which are more susceptible to crystalline perfection on annealing than crystallites that have higher stability (Jacobs et al., 1998a). The greatest post annealing changes in gelatinization temperatures have been observed in starches which had the lowest gelatinization temperatures in the native form, indicating that the extent of change occurred during annealing is affected by the extent of crystalline perfection in the native starch (Lan et al., 2008; Alvani, Qi, & Tester, 2012). The decrease in gelatinization temperature range reflects greater homogeneity or perfection of crystallites (Jacobs et al., 1998a; Jacobs & Delcour 1998; Tester et al., 2000) which restricts hydration/plasticization of the crystallites (Alvani et al., 2012). Gelatinization enthalpy (ΔH) has shown to remain unchanged or increase slightly after annealing. Since ΔH reflects the number of double helices that unravel and melt during gelatinization (Cooke & Gidley, 1992), no change in ΔH following annealing reflects that new double helices are not formed on annealing (Jayakody & Hoover, 2008; Lan et al., 2008). The increase in ΔH on annealing can be attributed to the increased amylose–amylose and/or amylose–amylopectin interactions, leading to the formation of new double helices (Tester et al., 2000; Waduge et al., 2006).

Annealing temperature, moisture content, and annealing time also affect gelatinization characteristics of starch. Studies have shown that more pronounced effects on gelatinization were observed when annealing temperature was set close but below $T_o$, however, if annealing temperature is very close to $T_o$, it would trigger gelatinization (Tester & Debon, 2000; Jayakody

& Hoover, 2008). Hoover and Vasanthan (1993) observed steep increase in gelatinization temperatures at an annealing moisture of 50% in wheat and lentil starches and at 10 and 70% moisture in potato and oat starches, respectively. A steep increase in ΔH occurred at moisture contents of 40 and 50% in potato and wheat starches, respectively; whereas changes in ΔH for oat and lentil starches were gradual (Hoover & Vasanthan, 1993). Increase of starch chain mobility with increasing moisture content allowing amylose-amylose and/or amylose-amylopectin interaction would explain the increase in gelatinization temperatures with increasing annealing moisture content (Jayakody & Hoover, 2008). Gelatinization temperatures increase with increasing annealing time, having more pronounced effects on $T_o$. The highest rate of increase in $T_o$ was observed in the first 30 minutes in wheat and waxy rice (Shi, 2008) and in the first 60 minutes in sweet potato (Genkina, Noda, Koltisheva, Wasserman, Tester, & Yuryev, 2003) and there after increase in $T_o$ slowed with increasing annealing time. Hoover and Vasanthan (1993) reported that annealing beyond 24 h, did not significantly increase gealtinization temperatures of oat, potato and lentil starches.

Susceptibility of annealed starches to acid hydrolysis has been studied in many reports, however, findings are not consistent due to variations in annealing method and starch source (Hoover & Vasanthan, 1993; Jacobs, Eerlingen, Rouseu, Colonna, & Delcour, 1998b; Tester et al., 1998; Nakazawa & Wang, 2003; Waduge et al., 2006). Hoover and Vasanthan (1993) reported a slight reduction (~5%) in hydrolysis on annealing in potato, lentil, oat, and wheat starches. Annealed normal wheat starch was more extensively degraded in the rapid phase of hydrolysis than its native counterpart, while during the slow phase of hydrolysis, there was no difference (Tester et al., 1998). In barley starches (normal, waxy and high amylose) the extent of decrease in acid hydrolysis on annealing has been shown to be less than 5% (Waduge et al., 2006). However,

Nakazawa and Wang (2003) reported that multistep annealing of wheat, tapioca, potato, maize, waxy maize and high amylomaize increased acid susceptibility.The extent of decrease in acid hydrolysis on annealing has been attributed to perfection of starch crystallites, increased resistance of α-D-(1→6) branch points, formation of V-amylose lipid complexes, and formation of amylose double helices (Hoover & Vasanthan, 1993; Jacobs et al., 1998b; Waduge et al., 2006). The increase in acid hydrolysis on annealing could be due to the production of void spaces as a result of crystalline perfection, which lead to porous structures and possible starch granule defects (Nakazawa & Wang, 2003).

Although there is a wide range of literature describing susceptibility of annealed starches towards enzyme hydrolysis, reported data are conflicting, which could be due to differences in annealing conditions, enzyme source, purity, and concentration, analysis methods and starch source (Jayakody & Hoover, 2008; BeMiller & Huber, 2015). Annealing has been shown to increase susceptibility of normal corn (Chung et al., 2009a; Chung, Liu & Hoover, 2009b; Rocha et al., 2012; O'Brien & Wang, 2008), waxy rice (Zeng et al., 2015), low-, medium-, and high-amylose rice (Dias, Zavareze, Spier, de Castro & Gutkoski, 2010), high amylose corn (Hylon V and Hylon VII) (O'Brien & Wang, 2008), waxy wheat (Lan et al., 2008), Oat (Hoover & Vasanthan, 1993), field pea, and lentil (Chung et al., 2009b, Chung, Liu & Hoover, 2010) and decrease susceptibility of waxy, normal and high amylose rice (Hung, Chau & Phi, 2016), normal wheat (Lan et al., 2008; Hoover & Vasanthan, 1993),and lentil (Hoover &Vasanthan, 1993) starches. Dias et al. (2010) found that the increase in the percentage of hydrolysis of low-, medium-, and high-amylose rice starches was more intense as the annealing temperature increased from 45 to 60$^{o}$C. O'Brien and Wang (2008) reported that the susceptibility of annealed potato starch towards bacterial α-amylase as well as amyloglucosidase was higher than its native

counterpart. However, annealing has been shown to reduce susceptibity of potato starch towards porcine pancreas α-amylase (Hoover & Vasanthan, 1993) and prancreatin (a mixture of α-amylase from porcine pancreatic stomach mucosa, lipaseand protease) (Jacobs, Eerlingen, Spaepen, Grobert & Delcour, 1998c). Rocha, Cunha, Jane, and Franco (2011) reported the susceptibility of potato starch towards bacterial α-amylase and amyloglucosidase was not changed upon annealing. Cassava starch showed a decrease in susceptibility towards porcine pancreas α-amylase (Jyothi, Sajeev, & Sreekumar, 2011), but an increase in susceptibility towards bacterial α-amylase and amyloglucosidase (Rocha et al.,2011) on annealing.

Slight changes were observed in RDS and RS contents while SDS content increased in normal maize starch on annealing (Chung et al., 2009a, 2009b; Wang et al., 2014). Increase in SDS content was attributed to slight disruption of crystalline lamellae or small dissociation of double helices (Wang et al., 2014). However, Chung et al. (2009b) reported an increase in RDS and RS contents and a decrease in SDS contents and Chung et al. (2009a) reported an increase in RDS levels and a slight decrease in SDS and RS levels in normal maize starch upon annealing. A decrease in RDS contents and increase in SDS and RS levels upon annealing were observed in waxy rice, normal rice and high amylose rice (Hung et al., 2016). However, Zeng et al. (2015) reported an increase in RDS contents and a decrease in SDS and RS contents of waxy rice upon annealing. RDS levels increased, SDS contents decreased and RS levels showed a slight increase in field pea and lentil starches upon annealing (Chung et al., 2009b). However, Chung et al. (2010) observed that annealing brought about an increase in RDS and SDS levels and a decrease in RS contents in field pea and lentil starches. Increase in RDS levels and a decrease in SDS and RS fractions on annealing could be due to the formation of porous structures (O'Brien & Wang, 2008) which allows greater accessibility of hydrolytic enzymes to penetrate into the granule

(Chung et al., 2009a). Enhanced interactions between starch chains could have caused an increase in RS levels (Chung et al., 2009b).

#### 2.9.1.4 Uses of annealed starches

Physical modifications such as annealing of starch are preferred over chemical modifications since it is simple, inexpensive, and especially it does not involve any chemical reagents (BeMiller & Huber, 2015). Since annealing enhances thermal stability and decrease set-back (Hoover & Vasanthan, 1993; Adebowale, Afolabi & Olu-Owolabi, 2005), annealed starches could be utilized in the canned and frozen food (Jayakody & Hoover, 2008). Hormdok and Noomhorm (2007) evaluated the quality of noodles when rice flour was substituted for rice starch modified by annealing and showed an improvement in the texture (adhesiveness, chewiness, and tensile strength) of noodles prepared with annealed rice starch, indicating that decrease in swelling power and amylose leaching and improved heat and shear stability upon annealing are desirable properties in noodle manufacturing (Jayakody & Hoover, 2008). An increase in resistant starch contents upon annealing was observed in waxy, normal and high amylose rice (Hung et al., 2016), normal corn, field pea, and lentil (Chung et al., 2009b) starches while maintaining granule structure. Resistant starch can be incorporated in foods without altering the appearance and texture, since it has a bland taste, white color and microparticulate structure (Jayakody & Hoover, 2008).

### 2.9.2 Acetylation

Acetylation is one of the widely used chemical modification of starch,which is achieved by esterification of native starch with either acetic anhydride or vinyl acetate in the presence of alkaline catalyst (eg: NaOH, KOH, $Ca(OH)_2$, $Na_2CO_3$) (Wang & Wang, 2002; Huang, Schols, Jin, Sulmann, & Voragen, 2007). Three free hydroxyl groups on $C_2$, $C_3$, and $C_6$ of the glucose

molecule of starch can be substituted during acetylation, and hence, theoretical maximum degree of substitution (DS) is 3 (Xu, Miladinov,&Hanna, 2004). In the presence of acetic anhydride and base catalyst, a nucleophilic substitution at an unsaturated carbon atom of acetic anhydride occurs (Fig 2.14) (Xu et al., 2004; Miyazaki, Van Hung, Maeda,& Morita, 2006). The three free OH groups have different reactivities, where the primary OH at $C_6$ is more reactive and is acetylated more readily than the secondary ones at $C_2$ and $C_3$ due to steric hindrance (Xu et al., 2004).

The DS and physicochemical properties depend on the starch source, type of acetylation reagent, reactant concentration, reaction medium (aqueous or anhydrous), pH, temperature and duration of reaction, and the presence of catalysts (Golachowski et al., 2015). Acetylated starches produced with acetic anhydride in the aqueous medium, and in the presence of alkaline catalyst were characterized by a DS (which measured based on the weight of acetyl groups introduced) around 0.2, which is permissible by regulations concerning the use of acetylated starch preparations in food products (Colussi et al., 2015; Golachowski et al., 2015), since starch acetate with DS 0.01 to 0.2 is approved by the FDA (Food and Drug Administration USA) for food applications (de Graaf, Broekroelofs, & Janssen, 1998). Acetic anhydride is permitted for use as a food additive in Canada at a level consistent with good manufacturing practice (GMP), which is the minimum amount needed to manufacture the modified starch.

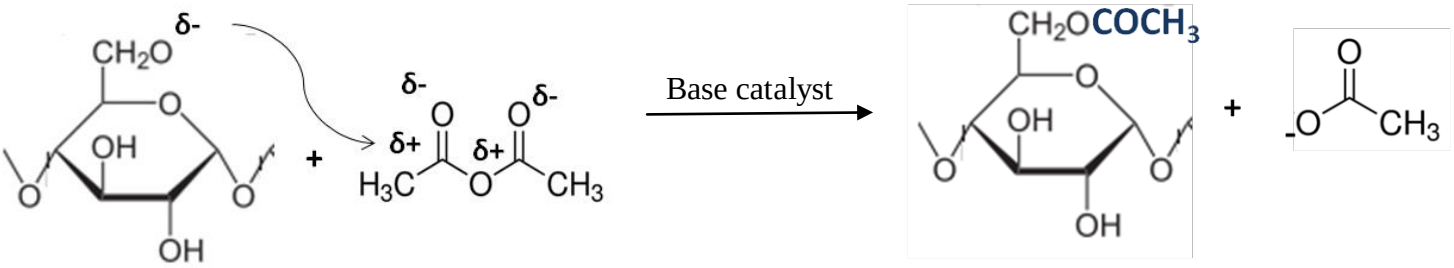



**Figure 2.14** Schematic representation of acetylation reaction of starch (Adapted from Xu et al., 2004)

#### 2.9.2.1 Impact of acetylation on morphology

Acetylation causes changes to granule morphology; however, the changes depend on starch source and DS. Studies have shown a relationship between the extent of morphological changes and the degree of substitution with acetyl groups; where starch with higher DS was characterized by greater morphological changes than starch with lower DS (Golachowski et al., 2015). Cracks and fissures were found in hulless barley starch and were more evident with advanced acetylation (from DS 0.029 to 0.085) (Chang & Lv, 2017). Sodhi and Singh (2005) examined several rice cultivars which achieved different DS ranging from 0.087 to 0.118 and reported that no significant differences were observed between the external morphology of native and acetylated rice starches, however, acetylation brought about slight aggregation of granules. Acetylation treatment brought granule fusion in corn and potato starches and the higher the DS (from DS 0.180-0.206 and 0.133-0.150, in potato and corn, respectively) greater the fused granules (Singh, Chawla, & Singh, 2004). Colussi et al. (2015) acetylated high amylose rice starch with different concentrations of acetic anhydride (5 g/100g, 10 g/100g and 20 g/ 100g, starch db) and found that the acetylation (DS 0.05-0.10) did not aggregate the granules and the granules did not show changes in the shape. However, a change in external morphology leading to surface roughness, formation of cavities, and fusion of few starch granules was observed upon acetylation (DS 0.050-0.081) of sorghum starch (Singh, Sodhi, & Singh, 2012).

#### 2.9.2.2 Impact of acetylation on starch structure

Changes in the granule structure upon acetylation have been studied using X-ray diffraction (Lawal & Adebowale, 2005; Xia, Wenyuan, Qianqian, Luqi, & Changxiao, 2011; Mbougueng, Tenin, Scher, & Tchiégang, 2012; Singh et al., 2012; Chang & Lv, 2017), Fourier transform infrared spectroscopy (FTIR) (Singh et al., 2004; Sodhi & Singh, 2005; Xia et al., 2011;

Mbougueng et al., 2012; Singh et al., 2012; Colussi et al., 2015; Sun, Zhang, & Ma, 2016; Chang & Lv, 2017), and nuclear magnetic resonance ($^1$H NMR) (Sun et al., 2016). Many studies have shown that greater structural changes occurred at a higher degree of substitution (Golachowski et al., 2015). X-ray diffraction patterns were not changed upon mild acetylation (DS<0.2) in sorghum (Singh et al., 2012), hulless barley (Chang & Lv, 2017), jack bean (Lawal & Adebowale, 2005), cassava and potato (Mbougueng et al., 2012) starches indicating that crystalline structure remained unchanged during acetylation. However, broader peaks in X-ray diffraction patterns were observed at higher DS (DS>1.5) reflecting the loss of crystallinity with increasing DS (Xia et al., 2011). Xia et al. (2011) postulated that acetylation reduces the formation of the inter- and intra-molecular hydrogen bonds in starch, and thereby resulted in the destruction of the ordered crystalline structure. The introduction of acetyl moiety in the acetylated starches has also been confirmed by the FTIR spectral analysis which shows the incorporation of acetyl groups in the starch molecule through a band assigned to carbonyl C=O (1730-1740 $cm^{-1}$) (Sodhi & Singh, 2005; Colussi et al. 2015; Sun et al., 2016; Chang & Lv, 2017). Sun et al. (2016) showed that the intensity of the band at 1731 $cm^{-1}$ increased with increasing DS (DS 0.056 to 0.092) in lotus rhizome starch. In comparison to the $^1$H NMR spectrum of native starch, new broad peaks at 2.01–2.08 ppm (which are corresponded to the protons of the acetyl groups) appeared in the spectrum of acetylated lotus rhizome starches and their integrations increased when the DS was changed from 0.056 to 0.092 (Sun et al., 2016).

#### 2.9.2.3 Impact of acetylation on starch properties

Acetylation increases water absorption, granule swelling, and solubility of starch. Introduction of acetyl groups facilitate the access of water to the amorphous areas, due to an intragranular structural disorganization caused by steric effects and reduction of the bond strength between

starch molecules (Gonzalez & Pérez, 2002; Ashogbon & Akintayo, 2014). The introduction of acetyl groups has shown to cause changes in thermal properties of starch which is generally studied by DSC (Golachowski et al., 2015). Acetylation reduced gelatinization transition temperatures and enthalpy of gelatinization in rice (Colussi et al., 2015), corn and potato (Singh et al., 2004), pinto bean, black bean, and navy bean (Hoover & Sosulski, 1985), cassava (Osundahunsi & Mueller, 2011), sorghum (Sing et al., 2012), and hulless barley (Chang & Lv, 2017) starches and the decrease in gelatinization temperatures and enthalpy increased with increasing DS. The decrease in gelatinization temperatures in acetylated starches shows that the introduction of voluminous groups to the chain enhances structure flexibility and starch will be vulnerable for water binding and leads to premature rupture of double helices during heating (Singh et al., 2012; Chang & Lv, 2017). Further, the acetyl groups may have also caused destabilization of granular structure, thus causing increase in swelling and decrease in gelatinization temperatures (Wootton & Bamunuarachchi, 1979). The decrease in the enthalpy of gelatinization in acetylated starches has been attributed to the disruption of double helices, present in crystalline and non-crystalline regions of the granule during the acetylation reaction (Colussi et al., 2015).

Studies showed that acetylation reduced retrogradation and syneresis of starch, and exhibited better transparency and clarity of gels (Golachowski et al., 2015). Starch retrogradation involves re-aggregation of starch molecules in stored gels, which involves hydrogen bonding for re-crystallization (Lawal & Adebowale, 2005). Acetyl groups prevent the alignment and association between starch chains as a result of electrostatic repulsion leading to reduction in retrogradation (Gonzalez & Pérez, 2002; Lawal & Adebowale, 2005). The reduction in syneresis in acetylated starches compared to native counterparts may be attributed to the introduction of acetyl groups.

Introduction of acetyl groups causes improvement in water retention capacity as a result of hindrance of inter-chain interaction between the macromolecules caused by acetyl groups (Sodhi & Singh, 2005; Singh et al., 2012). The increase in light transmittance percentage (high paste clarity) after the acetylation treatment can be explained by greater swelling of acetylated starch which allows more light to pass through the granules, instead of being reflected (Han et al., 2012). Inhibition of chain association upon acetylation favored the retention of an amorphous character and high clarity (Liu, Ramsden & Corke, 1997).

The acetylation process influences starch digestibility. Acetylation reduced RDS and SDS levels, while it increased RS content in hulless barley (Chang & Lv, 2017), rice (Xu et al., 2012), black bean and pinto bean (Simsek, Ovando-Martínez, Whitney, & Bello-Pérez, 2012). The different RDS levels reflects the interplay between the surface characteristics and the extent of molecular order at the granule surface while the SDS and RS differences can be accounted for crystalline stability and inner molecular order (Chang & Lv, 2017). Colussi et al. (2015) reported that different levels of acetylation did not change significantly the hydrolysis of rice starch subjected to the hydrolysis of α-amylase. The α-amylase digestibility of normal and waxy starches showed little change due to acetylation, whereas, high amylose was digested much more rapidly in the acetylated form (Liu et al., 1997). Acetylated pinto bean, black bean, and navy bean starches showed less susceptibility to α-amylase hydrolysis compared to native starches (Hoover & Sosulski, 1985). Digestibility of acetylated corn starch did not differ significantly at the early stage (60 min), but decreased at the later stage of hydrolysis (after 3 h) and it was postulated that the acetyl groups were inert to the enzymatic action, and thus provided the resistance of granule starch to enzymatic hydrolysis (Chung, Shin, & Lim, 2008). Xu et al. (2012) showed that the acetyl content had no obvious correlation to the RS content, since the RS content ranged from

53.98–69.45% when acetyl content ranged from 0.59% to 5.30%. The authors postulated that the reason might be that some acetyl groups, which bonded with the starch molecules could change the spatial structure of starch molecules, especially change the binding sites between α-amylase and starch molecule (Xu et al., 2012).

**2.9.2.4 Uses of acetylated starches**

There is a commercial interest in acetylated starches with a DS of 0.01-0.20 because of their use basedon properties to film formation, adhesion, thickening, stabilizing and texturizing (Boutboul, Giampaoli, Feigenbaum, & Ducruet, 2002). Acetylated starches have enhanced properties over their native counterparts and have been used for their stability and resistance to retrogradation (Wurzburg, 1986). The reduction in the pasting temperature is an advantage of acetylation because it allows the use of acetylated starches where a thickening agent must gelatinize at a lower temperature, and also acetylated starches are used to reduce energy costs during processing of products (Colussi et al., 2015). Acetylated starches are extensively used in a large variety of foods including baked goods, canned pie fillings, sauces, retorted soups, frozen foods, baby foods, salad dressings, and snack foods (Wurzburg, 1995). The increased RS content after acetylation demonstrates its nutritional value as a low glycemic index food as well as acetylated starches is of great nutritional potential as a dietary fiber (Chang & Lv, 2017). Acetylated starches with high DS have very different properties such as hydrophobicity, melt processability and are used in a number of non-food applications, such as tablet binders, hot melt adhesion, coating, cigarette filters, biodegradable packaging materials and pharmaceutical aspects (Ashogbon & Akintayo, 2014).

## 2.10 Analysis of starch structure using mass spectrometry

Mass spectrometry (MS) analysis involves the formation of gaseous ions from an analyte (M) and subsequent measurement of the mass-to-charge ration (*m/z*) of these ions (McLafferty & Turecek, 1993). The sample is converted to molecular or quasimolecular ions and their fragments depending on the ionization method used. Molecular ions are generally radical cations ($M^{+\bullet}$), formed by electron remove from $M^{\bullet}$, whereas, occasionally $M^{-\bullet}$ is produced for electronegative samples by adding electrons. Quasimolecular ions may be either positive or negative, such as $[M+H]^+$, $[M-H]^-$, $[M+Na]^+$, and $[M+ Cl]^-$ (Polce & Westdemiotis, 2002). Mass spectrometry is an important tool for the structural analysis of carbohydrates, which offers precise results, analytical versatility, and very high sensitivity (Zaia, 2004).

The "soft" mass spectrometric ionization techniques, such as fast atom bombardment (FAB), was used earlier to investigate oligosaccharides, however, over the past decades the FAB technique has been replaced by the more sensitive techniques of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) (Zaia, 2004; Mischnick, 2011). ESI and MALDI impart little energy to the sample, producing less fragmentation during the ionization process compared to FAB. Ions can be generated either in positive or negative ion mode, depending on the nature of the sample (Kailemia, Ruhaak, Lebrilla, & Amster, 2013). Number of difficulties can be encountered during the mass spectrometric analysis of carbohydrates. Carbohydrates commonly form sodium adducts at much lower yields and poly- or oligosaccharides show lower surface activity, exhibit higher polarity, are less stable, often show dispersity of molecular mass and chemical structure, and due to many stereochemical centers have isobaric ions, which cannot easily be differentiated by MS (Mischnick, 2011). Generally, starch is hydrolysed and/or derivatized before MS analysis to obtain better results.

In ESI, analyte ions are generated by passing a dilute solution (1−10 μM) through needle with a thin diameter placed near the mass spectrometer inlet capillary, at a potential of 1−4 kV. The potential difference between the tip and the capillary produces fine charged droplets, and then the solvent from the ions are vaporized by drying gas and high temperature as they are aspirated into the mass spectrometer (Karas, Bahr, & Dülcks, 2000). ESI can be combined with online liquid flow separation methods such as high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE) (Kailemia, 2013). Conventional ESI is known to be less effective for neutral oligosaccharides due to poor ionization efficiency. Permethylation of –OH, $-NH_2$, and –COOH groups in which a hydrogen atom is replaced with a methyl group enables improves the sensitivity for both neutral and acidic oligosaccharides and also it enables acidic oligosaccharides to be analyzed in positive mode where they are detected as metal adducts (Ruhaak et al., 2010; Wuhrer, 2013)

In general, MS structural analysis is based on tandem mass spectrometric ($MS^n$) experiments ($n \geq 2$). The major problem is to identify diagnostic product ions that enable discrimination among alternative structures (Čmelík & Chmelík, 2010). The low energy collision induced dissociation (CID) is the most commonly used fragmentation method, and has been implemented on various MS platforms (Kailemia, 2013). The CID fragmentation patterns differ according to the oligosaccharide sequence, size, and class of subunits, and are used to differentiate subtle structural differences and fragmentation of oligosaccharides have different features in the positive-ion and negative-ion modes (Zaia, 2004). In the positive ion mode, product ion scan also known as low energy collision induced dissociation CID-MS/MS of the precursor and $[M+Na]^+$ ions provide MS/MS spectra containing both glycosidic and cross-ring fragments (Domon & Costello, 1988; Harvey, Bateman, Bordoli, & Tyldesley, 2000). In the negative-ion mode, the

predominant ion derived from oligosaccharidesis $[M-H]^-$, but these ions frequently give rise to in-source fragments (Čmelík & Chmelík, 2010). Čmelík and Chmelík (2010) reported that negative-ion electrospray tandem mass spectrometry with an ion-trap provides a sensitive tool for sequence determination of oligosaccharides derived from starch, giving fragmentation of $[M-H]^-$ as well $[M+Cl]^-$ ions which allow an unambiguous differentiation between α-D-(1→4) and α-D-(1→6) linkages. Ionspray ionization and low energy CAD MS/MS analysis of the protonated molecules $[M+H]^+$ provided characteristic fingerprint patterns, and permitted differentiation of the various regioisomeric esters of sucrose (Chauvin, Thibault, Plusquellec, & Banoub, 1993). Asam and Glish (1997) showed that electrospray tandem mass spectrometry with quadrupole ion trap can be used to identify polysaccharide linkage by examining cross-ring cleavage product ions. Tandem MS experiments on an $^{18}O$-labeled disaccharide showed that the dissociation paths for Li and Na cationized species were the same and MS analysis of different oligosaccharides; trisaccharides (isomaltotriose, maltotriose, and panose), tetrasaccharide (isomaltotetrose) and pentasaccharide (maltopentaose) showed that tandem MS provides all available linkage information and $MS^n$ (n>2) can provide selected linkage information (Asam & Glish, 1997).

Starch is generally hydrolyzed by acid (van der Burgt et al., 1998, 1999, 2000) or enzymes such as isoamylase (Wang, Jiang, Vasanthan, & Sporns, 1999), α-amylase, β-amylase and amyloglucosidase (Chen, Schols, & Voragen, 2004) before MS analysis. Mass spectrometry has been rarely used to analyze the structure of native starch, whereas oligosaccharides are generally studied using MS. However, MS has been used to study the chemically derived starches which are used in both food and non food applications, since the number, location and distribution of substituents are important to find the relationship between structure and functional properties

(van der Burgt et al., 1998). FAB mass spectrometry study of methylated and acid hydrolysed potato starch showed that methylation in starch in granule suspension takes place preferably at the amorphous domains, whereas crystalline parts of amylopectin contains less methyl substituents (van der Burgt et al., 1999). Further, it was found that substituents in amylopectin were distributed heterogeneously, whereas substitution of amylose was almost random, indicating that crystalline lamellae are less accessible for substitution than amorphous branching points and amylose (van der Burgt et al., 2000). The mass results of the enzyme digested (by α-amylase and amyloglucosidase) hydrolysates determined by MALDI-time of flight (TOF)-MS showed that the enzyme-resistant fragments of acetylated potato amylose contain 1–3 acetyl groups in 3–7 glucose units, indicating that the acetyl groups were unevenly distributed in amylose (Chen et al., 2004).

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Materials

Waxy corn starch (STALEY® 7350 Waxy No.1) was obtained from A.E. Staley manufacturing company (Decatur, IL, USA). Waxy rice starch (Remyline AX-DR) was procured from A & B ingredients (Fairfield, NJ, USA). Waxy potato starch (ELAINE 100) was provided by National Starch Food Innovation (Bridgewater, NJ, USA). Waxy barley (CDC Candle) starch was obtained from the Department of Agricultural Food and Nutritional Science, University of Alberta, Canada. Pancreatin from porcine pancreas (cat. no. P-1625, activity 3× USP/g) was purchased from Sigma Chemical (St. Louis, MO, USA). Amyloglucosidase (EC 3.2.1.3., 3300/ml) from *Pseudomonas* sp and glucose oxidase-peroxidase assay kit were bought from Megazyme International Ireland Ltd. (Bray, Ireland). All chemicals and solvents were of ACS certified grade and used without any further purification.

## 3.2 Methods

### 3.2.1 Chemical composition of native starches

#### 3.2.1.1 Moisture content

The moisture content of the starch samples was determined according to the American Association of Cereal Chemists (AACC) (2000) method. Empty moisture pans were dried in an oven for 1 h at 130°C and cooled in a desiccator before the analysis. The moisture pans were pre-weighed and 5 g of starch samples were added and dried in an air forced oven at 130°C for 1 h. The samples were then removed and transferred to a desiccator. Samples were weighed after

they reached room temperature. Three replicates were used in each determination and the moisture content was determined as the percentage weight loss of sample using the following equation,

$$\text{Moisture (\%)} = \frac{(W_1 - W_2)}{(W_1 - W_0)} \times 100$$

Where,

$W_0$ - Weight of the moisture pan (g)

$W_1$ - Weight of sample and moisture pan before drying (g)

$W_2$ - Weight of sample and moisture pan after drying (g)

### 3.2.1.2 Phosphorous content

Phosphorous contents were quantified by inductively coupled plasma-optical emission spectrometry (ICP-OES) (Optima 3300 DV, Perkin Elmer, Waltham, MA, USA). Starch samples were prepared according to the method explained by Hong, Gomand, and Delcour (2015). Starch samples (0.05 g, dry basis) were weighed into glass screw-cap tubes and 1.0 ml 70% $HNO_3$ (v/v) was added. The tubes were sealed and incubated for 180 min at 140°C in an oil bath. Samples were then cooled and diluted to a final volume (10.0 ml) with MilliQ water. All measurements were performed in duplicates.

### 3.2.1.3 Apparent amylose content

Amylose content was determined since waxy starches may contain minute amounts of amylose, which can contribute to the changes in structural and functional properties. Amylose content of native waxy starches was determined in triplicates by the colorimetric method of Hoover and

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

### 3.2.2 Annealing

The physical modification technique used in this study was annealing. Starch samples were subjected to one step annealing. Native starch samples (10g, db) were weighed into glass containers and starch moisture content was brought to 75% by adding 30 ml of distilled water. The samples were sealed and heated at about 10°C below the onset temperature of gelatinization (onset temperature of gelatinization was determined for native waxy starches using differential scanning calorimetry as described in section 3.2.7.1) for different time periods (2, 4, 8, 16, 24, 48 and 72 h) in a thermostatically controlled water bath. After incubation, the suspensions were centrifuged (×2000 g) and supernatants were decanted. The annealed starches were washed three times with distilled water and once with acetone and air dried at room temperature.

### 3.2.3 Acetylation

#### 3.2.3.1 Preparation of acetylated samples

Waxy starches were chemically modified using mild acetylation technique. The method described by Phillips, Liu, Pan, and Corke (1999) was used to prepare acetylated starches. Starch (10 g, db) was dispersed in distilled water (22.5 ml) and stirred for 1 h at 25$^{o}$C. The pH of the suspension was adjusted to 8.0 with an aqueous NaOH (3%) solution followed by slow addition of 5, 10 and 20 g/100g of acetic anhydride (starch db), respectively, maintaining the pH within the range of 8.0–8.4 using 3% NaOH solution. The reaction was allowed to proceed for 15 min after the completion of acetic anhydride addition. The slurry was then adjusted to pH 4.5 with 0.5 M HCl to stop the reaction. The final suspension was centrifuged for 3 min at ×1000g and after sedimentation, it was washed free of acid, twice with distilled water and once with 95% ethanol, and then oven-dried at 40$^{o}$C.

#### 3.2.3.2 Determination of the acetyl percentage (Ac%) and degree of substitution (DS)

The percent acetylation (Ac%) and degree of substitution (DS) were determined titrimetrically following the method described by Wurzburg (1964). Acetylated starch (1.0 g, db) was placed in a 250 ml flask and 50 ml of 75% ethanol in distilled water was added. The flask was covered with aluminum foil, agitated in a water bath at 50$^{o}$C for 30 min, cooled and 40 ml of 0.5 M KOH was added. The slurry was kept under constant stirring at 200 rpm for 72 h. The excess alkali was back-titrated with 0.5 M HCl using phenolphthalein as an indicator. The solution was stood for 2 h, and then any additional alkali, which may have leached from the sample, was titrated. A blank, using the native unmodified starch, was also used.

$$\text{Ac \%} = \frac{[(V_{Blank}-V_{Smaple}) \times \text{Molarity of HCl} \times 0.043 \times 100]}{\text{Sample weight (db)}}$$

Where: $V_{Blank}$ and $V_{sample}$ were titration volumes in ml and sample weight was in g. DS was defined as the average number of sites per glucose unit that possess a substituent group (Whistler & Daniel, 1995).

$$DS = \frac{(162 \times \text{Ac \%})}{[4300 - (42 \times \text{Ac \%})]}$$

Where: 162 and 42 indicates molecular weight of a glucose molecule and an acetyl group, respectively.

### 3.2.4 Dual modification (Annealing-acetylation)

Annealed starch samples (10 g, db) prepared by the annealing procedure (Section 3.2.2) for 72 h time periodwere weighed into glass containers followed by acetylation (section 3.2.3.1) with 10 g/100g of acetic anhydride (starch db).

### 3.2.5 Granule morphology

#### 3.2.5.1 Scanning electron microscopy (SEM)

Granule morphologies of native, annealed, acetylated, and annealed-acetylated waxy starches were examined using FEI MLA 650 FEG (Brisbane, QLD, Australia) scanning electron microscope at an accelerating potential of 10 kV to evaluate the changes to granule surface characteristics that occurred upon modifications. The dried starch samples were spread thinly

onto round aluminum stubs with double-sided carbon adhesive tape and then carbon coated in a vacuum chamber.

### 3.2.6 Starch structure

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

The amylopectin branch chain length distribution of native starches was determined by isoamylase debranching of whole starch followed by HPAEC-PAD (Liu, Gu, Donner, Tetlow & Emes, 2007) in duplicates. Starch was dispersed in 2 ml of 90% dimethyl sulfoxide (DMSO- 5 mg/ml) by stirring in a boiling water bath for 20 min. Upon cooling, 6 ml of methanol were added and vortexed followed by placing the tubes in an ice bath for 30 min. The pellet, which was obtained by centrifugation (1000 x g for 12 min) was dispersed in 2 ml of 50 mM sodium acetate buffer (pH 3.5) by stirring in a boiling water bath for 20 min. After the equilibration of the tube at 37$^{o}$C,5 µl of isoamylase were added (EN102, 68,000 U/mg protein, Hayashibara Biochemicals Laboratories Inc., Okayama, Japan). The sample was incubated with slow stirring for 22 h at 37$^{o}$C and the enzyme was inactivated by boiling for 10 min. An aliquot (200 µl) of the cooled debranched sample was diluted with 2 ml of 150 mM NaOH. The sample was filtered in a 0.45 µm nylon syringe filter and then injected into the HPAEC-PAD system using a 50 µl loop. The HPAEC-PAD system consists of a Dionex DX 600 equipped with an ED50 electrochemical detector with a gold working electrode, GP50 gradient pump, LC30 chromatography oven, and AS40 automated sampler (Dionex Corporation). The standard triple potential waveform was used with the following periods and pulse potentials: T1 = 0.40s, with 0.20s sampling time, E1 = 0.05V; T2 = 0.20s, E2= 0.75V; T3= 0.40s, E3 = -0.15V. Data were obtained using Chromeleon

software, version 6.50 (Dionex Corporation, Sunnyvale, CA, USA). The weight fraction of DP <6, 6-12, 13-24, 25-36 and 37-58 was obtained from the area of peaks. The average chain length was also calculated. Eluents were prepared in distilled water with helium sparging: Eluent A was 500 mM sodium acetate in 150 mM NaOH, Eluent B was 150 mM NaOH. Separation of linear components was achieved on a Dionex CarboPac$^{TM}$ PA1 column with gradient elution (0 min, 40% eluent A; 5 min, 60% eluent A; 45 min, 80% eluent A) at a column temperature of 26$^{o}$C and a flow rate of 1 ml/min.

### 3.2.6.2 Fourier Transform Infrared (FTIR) spectroscopy

The infrared spectra of the native and modified starches were obtained in duplicates using a Fourier Transform Infrared (FTIR) spectrometer (Shimadzu Corporation, Kyoto, Japan) in the region of 4000-400 $cm^{-1}$ using a potassium bromide (KBr) disc containing 1% (w/w) finely ground sample. Ten readings were collected at a resolution of 4 $cm^{-1}$. FTIR spectra were obtained to determine the introduction of acetyl groups in the starch molecules.

### 3.2.6.3 Nuclear magnetic resonance (NMR) analysis

The $^{1}$H NMR spectra were recorded in duplicates using a Bruker AV300 spectrometer (Ettlingen, Germany) operating at 300 MHz. Native and modified waxy starches were dissolved in dueterated DMSO at 75$^{o}$C to obtain clear solutions. All analyses were carried out at 25$^{o}$C. $^{1}$H NMR spectra were obtained to determine the introduction of acetyl groups in the starch molecules.

#### 3.2.6.4 Wide angle X-ray diffraction (WAXS)

WAXS analysis of native and modified starches were performed in duplicates on a Rigaku Ultima IV X-ray diffractometer (Rigaku Americas, TX, USA) with operating conditions of target voltage 40 kV; current 44 mA; scanning range 3–35°; scan speed 1.00°/min; step time 0.95; divergence slit width 2/3°; scatter slit width 2/3°; sampling width 0.03°and receiving slit width 0.6 mm. The moisture content of the starches was adjusted to ~17% by being kept in a desiccator over saturated $K_2SO_4$ solutions ($a_w$=0.97) at room temperature for 21 days prior to WAXS analysis. Relative crystallinity was calculated using the IGOR pro 6.1 software (WaveMetrics Inc., Lake Oswego, OR, USA) and a Gaussian function was used for curve fitting. WAXS analysis was conducted to determine the impact of different modification techniques on crystalline packing and starch crystallinity.

### 3.2.7 Starch properties

#### 3.2.7.1 Differential scanning calorimetry (DSC)

Gelatinization parameters of native and modified starches were determined in triplicates using Mettler-Toledo differential scanning calorimeter (DSC1/700/630/GC200; Greifensee, Switzerland). Starch sample (3mg, db) and deionized water (11µl) were added into a DSC pan. The pan was sealed, reweighed and allowed to equilibrate overnight at room temperature prior to DSC analysis. The sample pan was heated from 25 to 120°C at a heating rate of 10°C/min with an empty pan as the reference. The transition temperatures reported are the onset ($T_o$), peak ($T_p$) and conclusion ($T_c$). The enthalpy of gelatinization ($\Delta H$) was assessed by integrating the area between the thermogram and a baseline under the peak and was expressed in terms of Joules per gram of dry starch.

### 3.2.7.2 Swelling factor (SF)

The swelling factor of native and modified starches when heated in the range 60-90°C in excess water was measured in triplicates according to the method of Tester and Morrison (1990a). Starch samples (50 mg, db) were weighed into screw cap tubes followed by addition of 5 ml distilled water and heating in a shaking water bath at an appropriate temperature for 30 min. The tubes were then cooled rapidly to 20°C in an ice water bath and 0.5 mL of Blue Dextran (Pharmacia MW 2x $10^6$, 5 mg/mL) was added. The contents were mixed by inverting the closed tubes several times. The tubes were centrifuged at 1500 x g for 10 min and the absorbance of the supernatant was measured at 620 nm. The absorbance of the reference tube that contained no starch was also measured. The SF is reported as the ratio of the volume of swollen granules to the volume of the dry starch, which was calculated by the following equation.

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

Where: $A_s$ and $A_r$ were absorbance of sample and reference at 620 nm, respectively. Sample weight (w) was in g.

### 3.2.7.3 Acid hydrolysis

Native and annealed waxy starches were hydrolyzed in triplicate with 2.2M HCl (1g, db, starch/40 ml) at 35°C in a water bath (New Brunswick Scientific, G76D, Edison, NJ, USA) for periods ranging from 1 to 35 days. Starch slurries were vortexed daily to re-suspend the deposited starch granules. Aliquots taken at specific time intervals were neutralized with 2.2M NaOH and centrifuged at 2000 x g for 10 min. The extent of hydrolysis was determined by

expressing the solubilized carbohydrates (Jane, & Robyt, 1984) as a percentage of the initial starch using the method of Bruner (1964).

#### 3.2.7.3.1 Determination of reducing value

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

### 3.2.7.4. *In-vitro* starch digestibility

*In-vitro* digestibility of native and modified starches was performed in triplicates by the AACC approved method 32–40 (AACC International, 2000). Starch sample (100 mg, db) was incubated with pancreatin (10 mg) and amyloglucosidase (12U) in 4 ml of 0.1 M sodium maleate buffer (pH 6.0) at 37°C with continuous shaking (200 strokes/min) for 0.5–72 h. At the end of incubation, 0.1 ml of aliquot was taken and 4 ml of 95% ethanol was added into it in order to terminate the enzyme reaction. The mixture was centrifuged (× 2000g) for 10 min. Glucose content of the supernatant was determined by a glucose oxidase-peroxidase assay (section

3.7.2.4.1) kit (Megazyme International Ireland Ltd, Bray, Ireland). Starch classification (Ambigaipalan, Hoover, Donner, & Liu, 2014) based on the rate of hydrolysis were: rapidly digestible starch (digested within 0.5 h; RDS), slowly digestible starch (digested between 0.5 and 16 h; SDS) and resistant starch (undigested after 16 h; RS), which was starch not hydrolyzed even after 16 h.

#### 3.2.7.3.1 Determination of glucose content by Megazyme glucose method

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

$$\text{Glucose } (\mu g/0.1 \text{ ml}) = \frac{\Delta A \text{ Sample}}{\Delta A \text{ Glucose standard (100 } \mu g)} \times 100$$

### 3.2.7.4 Retrogradation

#### 3.2.7.4.1 Turbidity

A 2% aqueous suspension of starch was heated in a boiling water bath for 1 h under continuous gentle stirring and then cooled for 20 min at 25°C. Turbidity was determined by measuring transmittance at 640 nm against water blank using a UV-visible spectrophotometer. The development of turbidity followed by storing the remaining starch pastes for 1 day at 4°C followed by 2-7 days at 25°C was measured. All measurements were done in triplicates.

#### 3.2.7.4.2 Differential scanning calorimetry (DSC)

DSC was used to measure retrogradation trasnsition temperatures and retrogradation enthalpies of native and modified starches. Melting of retrograded amylopectin was determined in triplicates by the method described by Jayakody, Hoover, Liu, and Donner (2007) with slight modifications. The samples were prepared with a starch to water ratio of 1:1. After the initial DSC run (section 3.2.7.1), sample pans containing the gelatinized starch were stored in the refrigerator at 4°C for 1 day followed by 6 days at 40°C. After days 1 and 7, the stored samples were equilibrated at 25°C for 1 h in a desiccator before reweighing and re-scanning. The scanning temperature range and heating rate were identical to that used for the study of gelatinization parameters (section 3.2.7.1).

### 3.2.8 Mass spectrometry of starch

#### 3.2.8.1 Sample preparation

##### 3.2.8.1.1 Permethylation

Permethylation was performed in triplicates according to the method described by Ciucanu and Costello (2003) with slight modifications. Starch sample (50 mg, db) was placed into a conical flask and stirred in 10 ml of DMSO and 100 µl of distilled water untildissolved. Finely powdered NaOH (4 equiv per mole of replaceable H) (0.4 g) was then added to the solution and the content of the flask was stirred vigorously for 30 min at room temperature to obtain a suspension. Methyl iodide (4 equiv per mole of replaceable H) (0.8 ml) was added and the mixture was stirred at room temperature for 2 h. The methylation reaction was quenched with water and neutralized with 1 M HCl. The permethylated product was extracted many times by the addition of the

chloroform (10.0 ml), shaking the mixture, and separating the layers by centrifugation. The combined chloroform layers were washed at least three times with distilled water and dried with dry nitrogen and under vacuum at 40°C. The residue containing the per-O-methylated sample was redissolved in 50% acetonitrile in distilled water and an aliquot was used for mass spectrometry analysis.

#### 3.2.8.1.2 Enzyme hydrolysis of starch

Hydrolyzed starch samples were prepared in triplicates by *in vitro* digestibility procedure (section 3.7.2.4) with 24 h hydrolysis time for waxy cereal starches and 7 days hydrolysis time for waxy potato starch. At the end of incubation, the enzymatic reaction was terminated by incubating at 95°C for 10 min. The sample was cooled to room temperature, centrifuged (1500 ×g, 15 min) and filtered in a 0.45 μm nylon syringe filter and an aliquot was used for mass spectrometry analysis.

### 3.2.8.2 Mass spectrometry

Electrospray ionization (ESI) mass spectra (positive-ion mode) were recorded with a Micromass Quattro quadrupole-hexapole-quadrupole mass spectrometer equipped with a megaflow ESI source. A personal computer (Compaq, PIII 300 MHzprocessor, running Windows NT 4, service pack 3) equipped with Micromass MASSLYNX 3.3 was used for mass acquisition and processing. The temperature of the ESI source was maintained at 75$^{o}$C. The operating voltage of the ESI capillary was 3.00 kV, and the high-voltagelens was set at 0.40 kV throughout the whole operation. ESI-MS spectra were recorded with a cone voltage of 25 V. Conventional ESI mass spectra were obtained by scanning in the Multi Channel Analysis mode (MCA) with a scan time of 1 s per 250 mass numbers. Spectra were an average of 20/30 scans. The mass scale was

calibrated in the positive-ion mode using a polyethylene glycol mixture. Component analysis of ESI-MS was performed using MASSLYNX algorithms which converta range of multiply charged cations into identifiable single components. ESI-MS with ‘‘maximum” entropy’’ (ESI-MS-MaxEnt) was performed using the Micromass MaxEnt Deconvolution (MMD) program. MaxEnt automatically disentangles the *m/z* spectrum produced by the mass spectrometer and presents the data for each individual starch hydrolysate analog in a single peak on a true molecular weight scale. Low-energy collisional dissociation tandem mass spectrometric (CID-MS/MS) experiments were conducted using the same instrument. Product-ion spectra of mass-selected precursor molecular-ion specieswere induced by collision with argon in the (r.f.-only) hexapole. The resulting product ions were analyzed by the second quadrupole. CID tandem mass spectra were recorded in an MCA mode and were consistently combined average of 25 scans for each type of precursor ion. A cone voltage varying from 20 to 35 V, collision energies varying from 10 to 40 eV and a collision gas pressure in the collision cell varying from $3.5x10^{-4}$ to $6.5x10^{-4}$mbar (1 bar = $10^5$ Pa) were used in all MS/MS experiments. The collision gas pressure was increased to induce the dissociation of the sodiated adductions (typical settings were around $6.0 \times 10^{-4}$ mbar). Precursor-ion MS/MS scans were obtained by scanning the first quadrupole while selecting a given *m/z* value with the second quadrupole.

**3.2.9 Statistical analysis**

Statistical analysis was performed among native and modified waxy starches. Native and modified starch samples were prepared in several batches and replicates were then taken from each batch. Analytical determinations for the samples were done in at least two replicates, mean values and standard deviations were reported. Analysis of variance (one way ANOVA) and

Turkey's HSD test ($p < 0.05$) was performed to determine significant differences among native and modified starches using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA)

# CHAPTER 4
# RESULTS AND DISCUSSION

## 4.1 Introduction

Waxy starches were modified using physical (annealing), chemical (acetylation), and combination of physical and chemical (annealing-acetylation) methods in order to examine whether the structural changes upon different modifications can enhance the desired attributes for food applications. The dual modification was compared with single modification techniques to evaluate the possibility of a novel modification technique. Structure of native and modified starches were analyzed using various techniques and the impact on different properties were evaluated in order to get a better understanding on how these various modification techniques affect the structure and properties of starches which are mainly composed of amylopectin.

## 4.2 Phosphorous and amylose contents of native waxy starches

Phosphorous content of native waxy corn (WC), waxy barley (WB), waxy rice (WR), and waxy potato (WP) are presented in Table 4.1. The presence of phosphate groups affects the physicochemical properties of starch, such as hydration and gel-forming capacity. The level of starch phophorylation varies considerably with the botanical origin (Blennow et al., 2002). Waxy potato contained the highest phosphorous content (0.075%) compared to waxy cereal starches (WB: 0.018%, WC: 0.007%, WR: 0.004%). In root and tuber starches, phosphorous is covalently linked to the starch as phosphate monoesters, whereas in cereal starches it occurs mostly as contaminating phospholipids (Lim & Seib, 1993; Gunaratne & Hoover, 2002). Previous studies have reported phosphorous contents of 0.006% in waxy corn (Tester et al., 2000), 0.024% in

waxy barley (Waduge et al., 2006), 0.07% in waxy potato (Varatharajan, Hoover, Liu, & Seetharaman, 2010), and 0.005% in waxy rice (Lim et al., 1994).

Waxy starches contain mainly amylopectin; there are differences in the amylose contents in the waxy starches depending on the starch source (variety and genotype). However, waxy starches contain only minute amounts of amylose. Apparent amylose content determination indicated that waxy starches used in this study were devoid any amylose. Previous studies have reported apparent amylose contents of 0.0-4.0 % in waxy barley (Waduge et al., 2006), 2.0% in waxy corn (Wang et al., 2014), and 0.0% in waxy wheat (Lan et al., 2008).

### 4.3 Amylopectin chain length distribution (APCLD) of native waxy starches

The branch chain length distribution of debranched amylopectin of native waxy starches are presented in Table 4.1. The chains were classified into five groups (DP <6, DP 6–12, DP 13–24, DP 25–36, DP >37). DP 6-12, DP 13-24, DP 25-36, and DP> 37 represent A, $B_1$-$B_3$ chains in amylopectin molecule, respectively (Hanashiro et al., 1996). The proportion of DP<6 chains was not significantly different ($p>0.05$) among starches, while the proportion of DP 6-12 chains was significantly lower ($p<0.05$) in WP starch. WP had a larger proportion of longer chains (DP >37), which contributed to the greater average chain length. WR showed the highest SC/LC ratio, which reflects a high degree of amylopectin branching. Similar values for proportion of A and $B_1$-$B_3$ chains of native waxy starches were observed for waxy corn (Kim, Kim, Moon, & Choi, 2014), waxy rice (Huang & Lai, 2014), waxy potato (Varatharajan et al., 2010) and barley (Song & Jane, 2000) starches.

**Table 4.1** Amylopectin chain length distribution and phosphorous content of native waxy starches[1]

| Starch source | Degree of polymerization (DPn) %[4] | | | | | | SC/LC[3] | Phosphorous content (%) |
|---|---|---|---|---|---|---|---|---|
| | DP<6 | DP 6-12 | DP 13-24 | DP 25-36 | DP >37 | $\overline{CL}$[2] | | |
| Waxy corn | 0.2±0.1[a] | 23.1±0.0[b] | 50.8±2.0[a] | 15.6±0.6[a] | 10.4±1.4[b] | 16.4±0.1[ab] | 0.303 | 0.007±0.01[c] |
| Waxy barley | 0.3±0.2[a] | 25.8±0.7[ab] | 48.4±2.9[a] | 15.2±0.4[a] | 10.4±1.6[b] | 16.0±0.1[b] | 0.353 | 0.018±0.02[b] |
| Waxy rice | 0.3±0.1[a] | 27.4±0.6[a] | 46.1±0.3[a] | 13.0±0.0[b] | 13.2±0.5[ab] | 15.7±0.2[b] | 0.383 | 0.004±0.03[c] |
| Waxy potato | 0.6±0.3[a] | 18.7±0.9[c] | 50.6±0.4[a] | 14.6±0.3[a] | 15.5±1.3[a] | 17.1±0.4[a] | 0.239 | 0.075±0.01[a] |

[1]All data represent the mean of duplicate determinations. Values followed by the same superscript in each column are not significantly different (p>0.05) by Tukey's HSD test.

[2]$\overline{CL}$ represents average chain length calculated by Σ (DPn × peak area)/Σ (peak area$_n$)

[3]SC/LC represents the proportion of short chains (DP<12) to long chains (DP>13).

[4] DPn: indicates degree of polymerization. The total relative area was used to calculate the percent distribution.

### 4.4 Evaluation of different annealing treatments based on gelatinization temperatures

Generally, excess moisture content (≥60–65%, w/w) and a temperature 5-15$^{o}$C below onset temperature of gelatinization are the annealing conditions which induce significant changes in a given starch (Tester & Debon, 2000; Jayakody & Hoover, 2008). It is important to ensure annealing treatment provides maximum crystalline perfection of starch before it is analyzed for the post annealing changes in structure and properties, since different starch sources, genotypes, or both exhibit diverse responses to the same annealing conditions (BeMiller & Huber, 2015). Therefore, in this study, starch to water ratio of 1:3 and annealing temperature of 10$^{o}$C below the onset temperature of gelatinization was used with different annealing time ranging from 2 to 72 h. The optimum conditions for annealing were determined based on the melting of crystallites which was measured concerning gelatinization temperatures of starch. Gelatinization temperatures of starch granules in water can be measured by differential scanning calorimetry (DSC).

The gelatinization transition temperatures (onset [$T_o$], midpoint [$T_p$] and conclusion [$T_c$]), gelatinization transition temperature range ($T_c$-$T_o$) as well as the enthalpy of gelatinization ($\Delta H$) of native and annealed starches are presented in Table 4.2. In all starches, $T_o$, $T_p$ and $T_c$ increased, and $T_c$-$T_o$ decreased with annealing time. It is well recognized that annealing increases gelatinization transition temperatures and narrows temperature range as a result of the perfection of existing crystallites (Jayakody & Hoover, 2008). The increases in $T_o$ in the first 2 h were 1.6, 1.7, 4.3, and 5.1°C for WC, WR, WB, and WP, respectively. The rate of increase in $T_o$ slowed down with increasing annealing time after 2 h. Similar results were observed for wheat and waxy rice starches, where the highest rate of increase in $T_o$ was observed in the first 30 min (Shi, 2008). Maximum gelatinization transition temperature values were observed after 72 h of

annealing. Even though there was no significant difference ($p>0.05$) between the gelatinization transition temperatures at 48 and 72 h of annealing (except for $T_c$ in WP and WB), to ensure maximum crystalline perfection in all the tested starches, 72 h was selected for the annealing time for the rest of the study.

**Table 4.2** Gelatinization parameters of native and annealed waxy starches[1]

| ANN time (h) | Gelatinization transition parameters[2] | | | | |
|---|---|---|---|---|---|
| | $T_o$ (°C)[3] | $T_p$ (°C)[3] | $T_c$ (°C)[3] | $T_c$-$T_o$ (°C)[4] | ΔH (J/g)[5] |
| **Waxy corn** | | | | | |
| Native | 64.2±0.0$^{e}$ | 70.3±0.3$^{d}$ | 76.3±0.1$^{c}$ | 12.1±0.0$^{a}$ | 14.8±0.2$^{ab}$ |
| 2 | 65.8±0.0$^{d}$ | 71.0±0.1$^{cd}$ | 77.3±0.1$^{bc}$ | 11.5±0.1$^{b}$ | 14.0±0.1$^{abcd}$ |
| 4 | 66.4±0.0$^{d}$ | 71.3±0.1$^{bc}$ | 77.5±0.1$^{abc}$ | 11.1±0.1$^{bc}$ | 13.8±0.2$^{bcd}$ |
| 8 | 67.1±0.1$^{c}$ | 71.9±0.0$^{b}$ | 77.9±0.4$^{ab}$ | 10.8±0.3$^{c}$ | 13.6±0.3$^{cd}$ |
| 16 | 67.7±0.3$^{bc}$ | 72.0±0.5$^{b}$ | 77.6±0.6$^{ab}$ | 9.9±0.3$^{d}$ | 13.7±0.1$^{cd}$ |
| 24 | 67.8±0.2$^{b}$ | 72.0±0.0$^{b}$ | 77.4±0.3$^{abc}$ | 9.6±0.1$^{d}$ | 13.2±0.4$^{d}$ |
| 48 | 69.8±0.2$^{a}$ | 73.7±0.3$^{a}$ | 78.4±0.1$^{a}$ | 8.6±0.1$^{e}$ | 15.0±0.3$^{a}$ |
| 72 | 69.9±0.0$^{a}$ | 73.7±0.0$^{a}$ | 78.3±0.1$^{ab}$ | 8.4±0.1$^{e}$ | 14.6±0.4$^{abc}$ |
| **Waxy barley** | | | | | |
| Native | 56.9±0.1$^{f}$ | 61.4±0.0$^{g}$ | 67.1±0.1$^{f}$ | 10.2±0.1$^{a}$ | 11.9±0.1$^{a}$ |
| 2 | 61.2±0.1$^{e}$ | 63.9±0.1$^{f}$ | 68.1±0.1$^{e}$ | 6.9±0.1$^{b}$ | 11.4±0.2$^{a}$ |
| 4 | 62.1±0.2$^{de}$ | 64.5±0.0$^{ef}$ | 68.8±0.0$^{d}$ | 6.7±0.3$^{bc}$ | 11.7±0.3$^{a}$ |
| 8 | 62.7±0.1$^{cd}$ | 65.1±0.1$^{de}$ | 69.4±0.0$^{c}$ | 6.7±0.1$^{bcd}$ | 11.3±0.1$^{a}$ |
| 16 | 63.3±0.3$^{bc}$ | 65.6±0.1$^{cd}$ | 69.7±0.2$^{bc}$ | 6.3±0.0$^{bcd}$ | 11.9±0.4$^{a}$ |
| 24 | 63.9±0.1$^{ab}$ | 66.2±0.0$^{bc}$ | 69.8±0.0$^{bc}$ | 5.9±0.1$^{de}$ | 11.8±0.2$^{a}$ |
| 48 | 64.9±0.7$^{a}$ | 67.1±0.6$^{a}$ | 70.9±0.3$^{a}$ | 6.0±0.4$^{cde}$ | 11.9±0.1$^{a}$ |
| 72 | 64.6±0.0$^{a}$ | 66.7±0.1$^{ab}$ | 70.0±0.2$^{b}$ | 5.4±0.1$^{e}$ | 12.0±0.2$^{a}$ |
| **Waxy rice** | | | | | |
| Native | 59.1±0.2$^{f}$ | 66.5±0.2$^{d}$ | 72.8±0.3$^{c}$ | 13.8±0.4$^{a}$ | 11.3±0.1$^{ab}$ |
| 2 | 60.8±0.0$^{e}$ | 66.9±0.1$^{cd}$ | 73.1±0.1$^{bc}$ | 12.3±0.1$^{b}$ | 11.0±0.4$^{b}$ |
| 4 | 61.3±0.1$^{de}$ | 67.2±0.1$^{bcd}$ | 73.1±0.2$^{bc}$ | 11.8±0.1$^{bc}$ | 12.3±0.4$^{a}$ |
| 8 | 62.0±0.0$^{cd}$ | 67.6±0.1$^{bc}$ | 73.8±0.1$^{ab}$ | 11.8±0.1$^{bc}$ | 12.0±0.2$^{a}$ |
| 16 | 62.4±0.2$^{bc}$ | 67.7±0.2$^{bc}$ | 73.8±0.0$^{ab}$ | 11.4±0.1$^{c}$ | 12.0±0.3$^{a}$ |
| 24 | 62.8±0.2$^{b}$ | 68.0±0.0$^{b}$ | 74.0±0.2$^{ab}$ | 11.2±0.0$^{c}$ | 11.8±0.1$^{ab}$ |
| 48 | 64.9±0.0$^{a}$ | 69.3±0.0$^{a}$ | 74.0±0.0$^{ab}$ | 9.1±.0.0$^{d}$ | 11.6±0.3$^{ab}$ |
| 72 | 65.5±0.4$^{a}$ | 69.8±0.5$^{a}$ | 74.6±0.5$^{a}$ | 9.1±0.1$^{d}$ | 11.7±0.1$^{ab}$ |

**Table 4.2** *(continued)*

| ANN time (hours) | Gelatinization transition parameters[2] | | | | |
|---|---|---|---|---|---|
| | $T_o$ ($^oC$)[3] | $T_p$ ($^oC$)[3] | $T_c$ ($^oC$)[3] | $T_c$-$T_o$ ($^oC$)[4] | $\Delta H$ (J/g)[5] |
| **Waxy potato** | | | | | |
| Native | 62.9±0.5$^{e}$ | 68.3±0.5$^{f}$ | 73.6±0.2$^{e}$ | 11.1±0.2$^{a}$ | 17.5±0.1$^{abc}$ |
| 2 | 68.0±0.2$^{d}$ | 71.4±0.1$^{e}$ | 76.3±0.1$^{d}$ | 8.3±0.0$^{b}$ | 17.0±0.0$^{abcd}$ |
| 4 | 68.5±0.1$^{cd}$ | 71.7±0.1$^{de}$ | 76.4±0.2$^{d}$ | 7.9±0.1$^{b}$ | 16.8±0.2$^{bcd}$ |
| 8 | 69.2±0.1$^{bc}$ | 72.4±0.1$^{cd}$ | 76.7±0.1$^{cd}$ | 7.5±0.1$^{c}$ | 16.5±0.0$^{cd}$ |
| 16 | 69.3±0.0$^{b}$ | 72.9±0.1$^{bc}$ | 76.7±0.1$^{cd}$ | 7.4±0.1$^{c}$ | 16.2±0.1$^{d}$ |
| 24 | 69.8±0.1$^{b}$ | 72.9±0.1$^{bc}$ | 77.3±0.1$^{bc}$ | 7.5±0.0$^{c}$ | 16.7±0.6$^{cd}$ |
| 48 | 70.7±0.1$^{a}$ | 73.7±0.1$^{ab}$ | 77.6±0.3$^{b}$ | 6.9±0.1$^{d}$ | 17.9±0.3$^{a}$ |
| 72 | 71.0±0.0$^{a}$ | 73.9±0.0$^{a}$ | 78.3±0.0$^{a}$ | 7.3±0.1$^{cd}$ | 17.8±0.3$^{ab}$ |

[1]All data represent the mean of triplicates. Values followed by the same superscript in each column in each starch source are not significantly different ($p>0.05$).

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

[3]$T_o$, $T_p$, and $T_c$ indicate the onset, peak, and end temperature of gelatinization, respectively.

[4]($T_c$-$T_o$) indicates the gelatinization temperature range.

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

### 4.5 Evaluation of different acetylation treatments based on the degree of substitution

The applicability of acetylated starches is completely dependent on the degree of substitution (DS) which depends on many factors. However, starch acetate with an acetyl percentage (Ac%) less than 2.5g/100g or DS 0.01 to 0.2 is approved by the FDA (Food and Drug Administration USA) for food applications (de Graaf et al., 1998; Colussi et al., 2015). In this study different acetic anhydride levels were used and Ac% and DS were determined to evaluate whether starch acetates have acceptable DS for food application. The Ac% and DS of acetylated waxy starches are given in Table 4.3. The Ac% of acetylated WC, WR, WB, and WP ranged from 0.82-2.29, 0.89-4.81, 1.56-5.63, and 0.23-1.92%, respectively. The DS of acetylated WC, WR, WB, and WP ranged from 0.03-0.09, 0.03-0.19, 0.06-0.22, and 0.01-0.07, respectively. The acetylated starches with the highest content of acetic anhydride (20g/100g) had higher Ac% and DS compared to starch acetylated with 5 g/100g acetic anhydride. At the highest content of acetic anhydride, WC and WP starches had Ac% less than 2.5%, which is less than FDA recommended value. However, Ac% of WB and WR reached slightly above 2.5%. Therefore, acetylation treatment was not conducted beyond acetic anhydride content of 20g/100g. The difference in the Ac% and DS of waxy starches, under similar acetylation conditions, may be attributed to differences in the granule structure. Low DS values would be associated with the higher magnitude of the associated bonding between starch chains, which restrict penetration of acetyl groups (Hoover & Sosulski, 1985).

Luo and Shi (2012) observed that the DS of acetylated waxy corn starch varied from 0.30 to 1.08, when reaction time varied from 40 to 240 min. Even though literature data are scare for acetylation of waxy starches, many studies have investigated acetylation of starches with varying amylose content. The DS and Ac% of acetylated rice starch ranged from 0.05 to 0.10, and 1.37-

2.58%, respectively, when 5, 10, and 20g/100g acetic anhydride concentrations were used (Colussi et al., 2015). The DS and Ac% of acetylated hulless barley starches were 0.029, 0.059, 0.085, and 0.765, 1.53, 2.20 with the acetic anhydride addition of 3.75, 7.5, 11.25 g/100 g, respectively (Chang & Lv, 2017). Singh et al. (2004) reported Ac% ranging from 4.68-5.20 and 3.43-3.74, in potato and corn starches, respectively, while DS ranged from 0.180-0.206 in potato and 0.133-0.150 in corn starch with six different acetic anhydride contents of 2-12g/100g. Differences in Ac% and DS can be attributed to the concentration, pH, reaction time, botanical origin and variations in granule size and structure (Colussi et al., 2015).

Dual modification of waxy starches was conducted with 10g/100g acetic anhydride after 72 h of annealing treatment since 10g/100g is the midpoint of the three contents of acetic anhydride used and it would give a better understanding about how structural changes occurring upon annealing would affect the acetylation process. Dual modification treatment (ANN-Ac) resulted in a significant increase in Ac% and DS in all waxy starches indicating that structural changes occurred during annealing facilitates the introduction of acetyl groups to the starch chains. During annealing, the plasticizing effect of water increases glucan chain mobility within the amorphous lamella regions of the semicrystalline growth ring and further annealing entails the movement of complete molecular sequences within a crystalline lattice (Jayakody & Hoover, 2008). The increased mobility of amylopectin chains and subsequent structural changes occurring on annealing would have created more space for acetyl group introduction during dual modification treatment. Since the DS of ANN-Ac starches were higher than DS of acetylated starches with 20% acetic anhydride; it showed that greater substitution could be achieved with less initial acetic anhydride content when using dual modification.

**Table 4.3** Percentage of acetyl groups (Ac%) and degree of substitution (DS) of waxy starches acetylated with different concentrations of acetic anhydride[1]

| Acetic anhydride (g/100g)[2] | Waxy corn | | Waxy rice | | Waxy barley | | Waxy potato | |
|---|---|---|---|---|---|---|---|---|
| | Ac% | DS | Ac% | DS | Ac% | DS | Ac% | DS |
| 5 | 0.82±0.04[d] | 0.03±0.00 [d] | 0.89±0.03[d] | 0.03±0.00[d] | 1.66±0.07[d] | 0.06±0.00[d] | 0.23±0.02[c] | 0.01±0.00[c] |
| 10 | 1.51±0.09[c] | 0.06±0.00[c] | 1.35±0.09[c] | 0.05±0.00[c] | 3.12±0.11[c] | 0.12±0.00[c] | 0.42±0.07[c] | 0.02±0.00[c] |
| 20 | 2.29±0.08[b] | 0.09±0.00[b] | 4.81±0.26[b] | 0.19±0.01[b] | 5.63±0.17[b] | 0.22±0.01[b] | 1.92±0.11[b] | 0.07±0.00[b] |
| Ann-Acetyl 10 | 3.28±0.11[a] | 0.13±0.00[a] | 6.12±0.08[a] | 0.25±0.00[a] | 6.10±0.09[a] | 0.24±0.00[a] | 3.21±0.19[a] | 0.12±0.01[a] |

[1]All data represent the mean of triplicates. Values followed by the same superscript in each column are not significantly different ($p>0.05$).

[2]Concentration of acetic anhydride used in the reaction (starch basis)

### 4.6 Impact of annealing, acetylation, and annealing-acetylation on granule morphology

Scanning electron microscopy (SEM) images showed that WC consisted of a mixture of small spherical and large polyhedral granules, while WB had large spherical, disc shaped and small irregular shaped granules. WP starch had large round or oval granule shapes, and WR starch consisted of small polyhedral granules (Fig. 4.1a-d). The granule surfaces of native WP and WR starches appeared to be smooth with no evidence of pores, indentations or fissures. However, pores were present on the surface of native WC and WB starch granules. Presence of pores was also observed on the surface of wheat, rye, barley, sorghum, millet, and corn starches (Fannon et al., 1992; Waduge et al., 2006; Wang et al., 2014).

Annealing did not induce significant changes on the granule surface of waxy starches (Fig. 4.1e-h). Wang et al. (2014) have also shown that granule surface characteristics of waxy corn remain unchanged upon annealing. However, Waduge et al. (2006) observed a slight increase of pore size in waxy barley and Rocha et al. (2012) reported an increase in the number of pores in waxy corn upon annealing.

Acetylation caused slight changes to the morphology of granules, and these changes were more evident with increasing DS. Rough granule surface, slight fragmentation, and indentation were observed in all waxy starches upon acetylation (Fig 4.2a-l). Hong, Zeng, Buckow and Han (2018) observed defects and bumps on the granular surface of waxy corn starch without obviously deformed shape upon acetylation (6g/100g of acetic anhydride). Acetylation brought granule fusion in corn and potato starches and more fused granules were observed with increasing DS (Singh et al., 2004). Sodhi and Singh (2005) observed slight aggregation of rice starch granules upon acetylation. However, granule fusion was not observed in acetylated waxy starches in the current study. Similarly, aggregation of the granules and change in the granule

shape were not observed for acetylated rice starch (Colussi et al., 2015). Surface roughness, formation of cavities, and fusion of few starch granules were observed upon acetylation of sorghum starch, and these changes may be attributed to the gelatinization of the surface of starch granules with sodium hydroxide (NaOH) during the acetylation treatment (Singh et al., 2012).

Even though annealing did not cause changes to granule morphology, acetylation of annealed starches with 10% acetic anhydride had caused slight morphological changes (Fig 4.2m-p) which were similar to acetylated waxy starches. However, acetylation and dual modification did not induce significant changes to the size and shape of the granules, indicating that granule integrity was maintained upon starch modification.

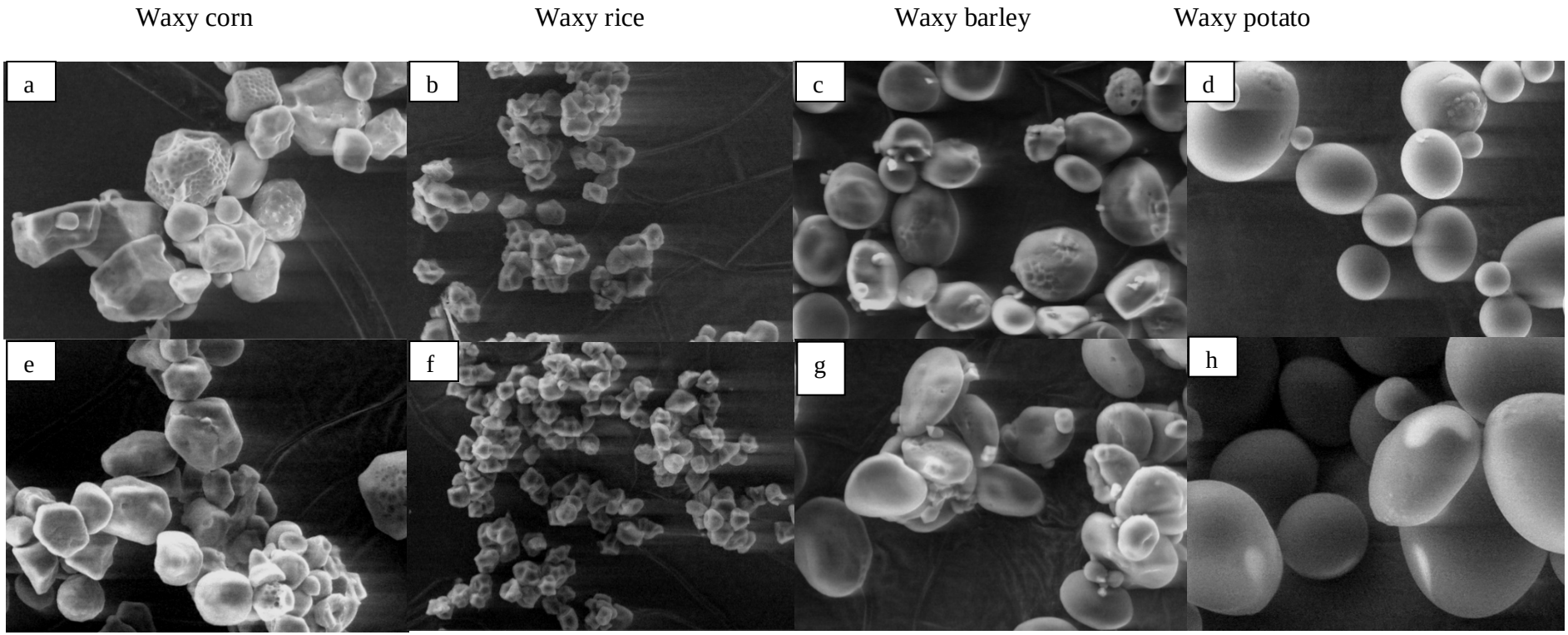



**Figure 4.1.** Scanning electron microscopy (×2500) of native (a-d) and annealed (e-h) waxy starches

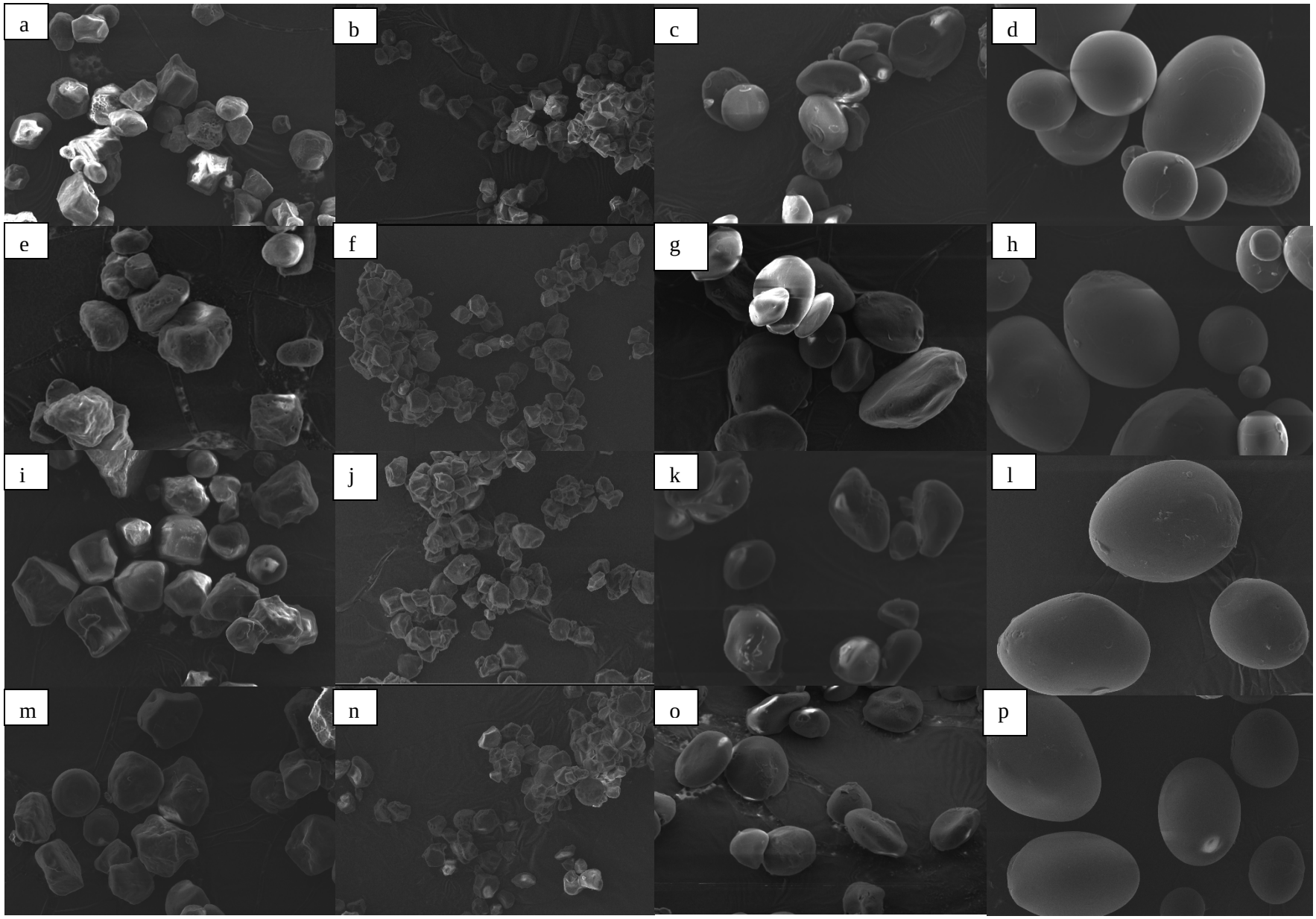



**Figure 4.2** Scanning electron microscope (×2500) of acetylated with 5 (a-d), 10 (e-h), and 20% acetic anhydride (i-l) and annealed-acetylated (m-p) waxy starches

## 4.7 Impact of annealing, acetylation, and annealing-acetylation on starch structure

### 4.7.1 Fourier transform infrared (FTIR) spectroscopy

FTIR spectra were used to analyze structural changes in acetylated waxy starches. Spectra of native waxy starches (Fig 4.3) showed an extremely broad band at 3366-3472 $cm^{-1}$, resulting from the vibration of the hydroxyl group (O–H), bands at around 2932 $cm^{-1}$ and 1644-1663 $cm^{-1}$, corresponding to the C–H vibration stretch and tightly bound water in the starch, respectively (Diop, Li, Xie, & Shi, 2011). Several discernible bands at 1159, 1081, 1015 $cm^{-1}$, which were attributed to C–O bond stretching and characteristic absorption bands at 929, 861, 766, 710 $cm^{-1}$ due to the entire glucose ring stretch vibrations (Chi et al., 2008) were also observed in the spectra of native waxy starches. Annealing did not cause changes to FTIR spectra of waxy starches since annealing did not introduce any new bonds in the starch structure.

FTIR spectra of acetylated and dual modified WC, WB, and WR starches (Fig 4.4a-c) showed some new absorption bands at around 1737, 1375, 1252 $cm^{-1}$ assigned to carbonyl C=O, $CH_3$ symmetry deformation vibration and carbonyl C–O stretch vibration, respectively (Chi et al., 2008). Acetylated and dual modified WP starched showed bands at only 1737 and 1252$cm^{-1}$ (Fig 4.4d). These new absorption bands suggest that the acetylated starch products were formed during the esterification process as well as dual modification (Chi et al., 2008). An introduction of acetyl moiety through a band at 1735.8 and 1730.8$cm^{-1}$ was observed for acetylated sorghum (Singh et al., 2012) and rice starches (Sodhi & Singh, 2005). New absorption bands at 1742, 1435, 1375, 1240 $cm^{-1}$ were observed in acetylated rice starches (Colussi et al., 2015).

The intensity of the band at around 1737$cm^{-1}$ increased with increasing DS of acetylated waxy starches indicating the presence of a higher number of acetyl groups. Chang and Lv (2017)

observed bands at 1245 and 1730 $cm^{-1}$ in acetylated hulless barley starches, and certain peaks displayed more intense, as DS increased (from DS 0.029 to 0.085). The absence of the peaks in the region 1850-1760 $cm^{-1}$ and 1700 $cm^{-1}$ implied that the product was free of unreacted acetic anhydride and acetic acid byproducts, respectively (Diop et al., 2011; Colussi et al., 2015).

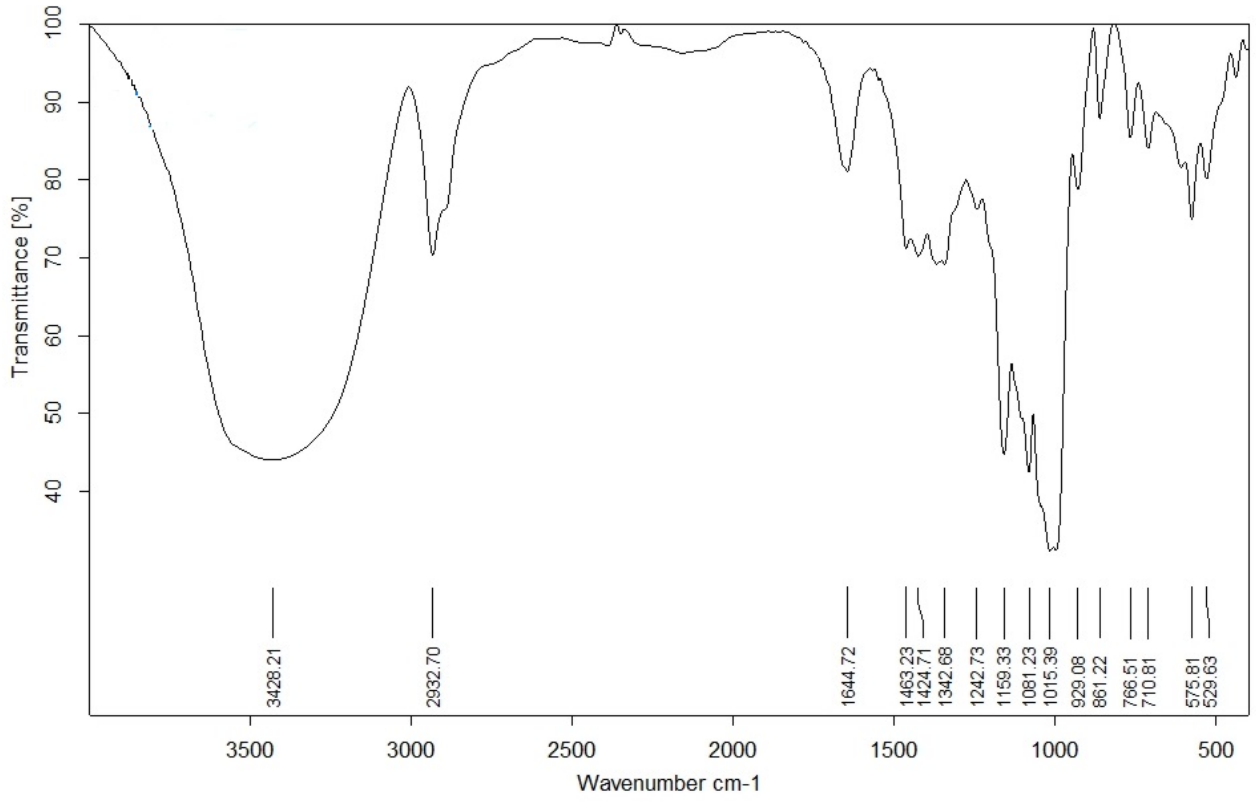



**Figure 4.3** FTIR spectrum of native waxy barley starch

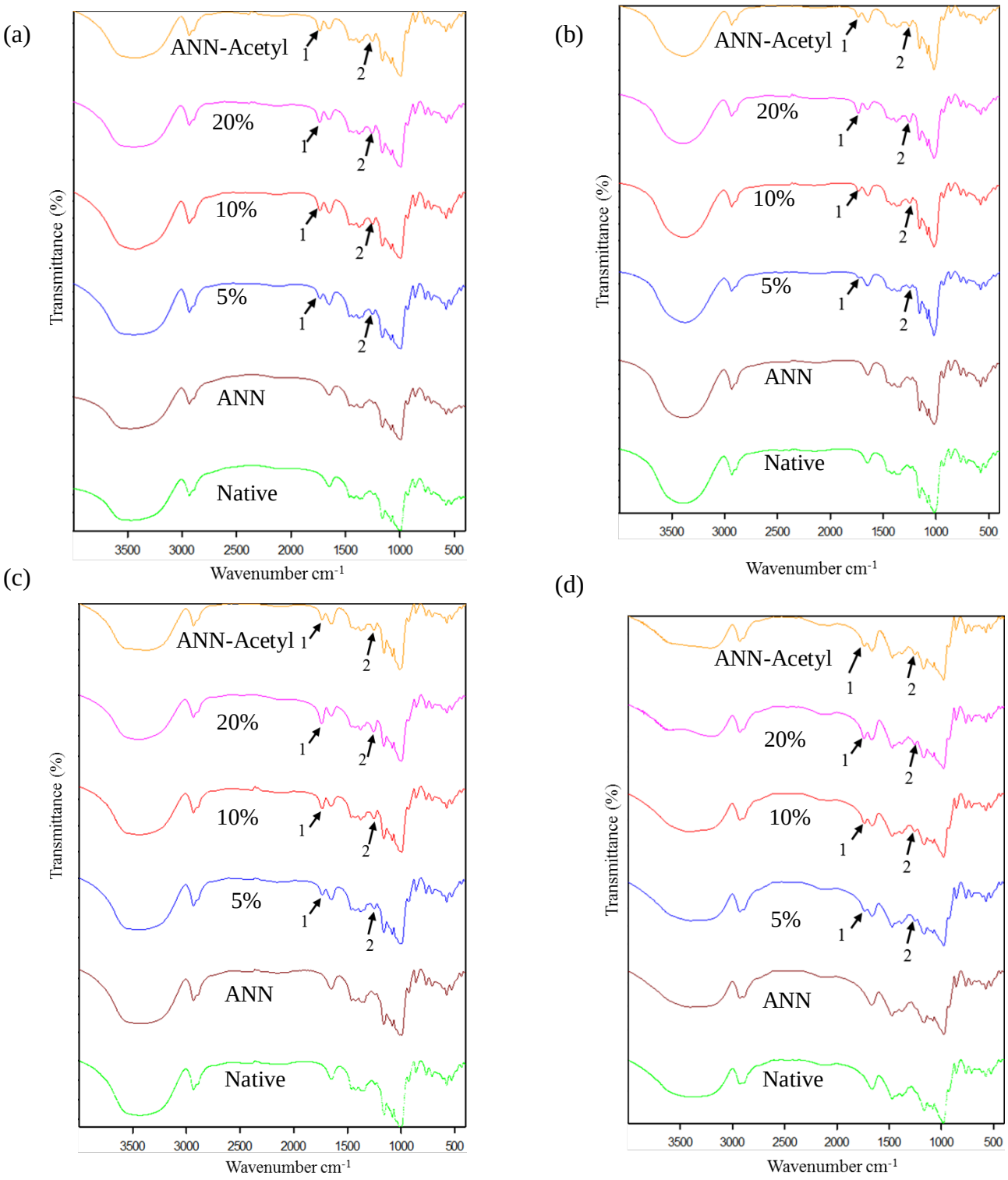



**Figure 4.4** FTIR spectra of native, annealed (ANN), acetylated (with 5, 10, and 20% acetic anhydride), and annealed-acetylated waxy corn (a), waxy rice (b), waxy barley (c), and waxy potato (d) starches. Arrow 1 and 2 indicate bands at around 1737 $cm^{-1}$ and 1252 $cm^{-1}$, respectively.

### 4.7.2 Nuclear magnetic resonance ($^1$H NMR) spectroscopy

The $^1$H NMR spectra of native starch showed chemical shifts which can be assigned as follows: 5.10 ppm to H-1, 5.39 ppm to OH-2, 5.48 ppm to OH-3, 4.56 ppm to OH-6, and 3.32-3.62 ppm (Fig 4.5) to protons of glucose unit (Chi et al., 2008). Annealing did not cause changes to $^1$H NMR spectra of waxy starches since annealing did not introduce any new bonds in the starch structure.

In comparison to the spectrum of native waxy starches, new broad peaks at 2.01–2.06 ppm appeared in the spectra of acetylated waxy starches (Fig 4.6). These peaks corresponded to the protons of the acetyl groups, and their integrations increased when the DS values increased. Integration values of protons of acetyl groups in acetylated starches with 20% acetic anhydride were 0.22, 0.29, 0.38, and 0.19 for WC, WR, WB, and WP, respectively. The characteristic peaks for hydroxyl groups of the glucose unit were still observed in acetylated waxy starches, indicating that only a portion of hydroxyl groups participated in the esterification reaction (Chi et al., 2008). Similar results were reported in acetylated lotus rhizome starches, where integration of protons of acetyl groups increased when DS value was changed from 0.056 to 0.092 (Sun et al., 2016). Dual modification of waxy starches also showed the appearance of peaks at 2.01–2.06 ppm with integration values of 0.21, 0.21, 0.20, and 0.16 for WC, WR, WB, and WP respectively.

The methyl proton signals of the acetyl groups placed at $C_6$, $C_2$ and $C_3$ of the acetylated waxy starches were identified at around 2.05, 2.03, and 2.00 ppm (Fig 4.5), respectively (Zięba, Szumny, & Kapelko, 2011; Sun et al., 2016). The peak at 2.05 ppm was evident in all acetylated and dual modified waxy starches, however, appearance of other the two peaks varied depending

on the DS and starch source, indicating that the primary OH at $C_6$ is more reactive and is acetylated more readily than the secondary ones at $C_2$ and $C_3$ due to steric hindrance (Xu et al., 2004).

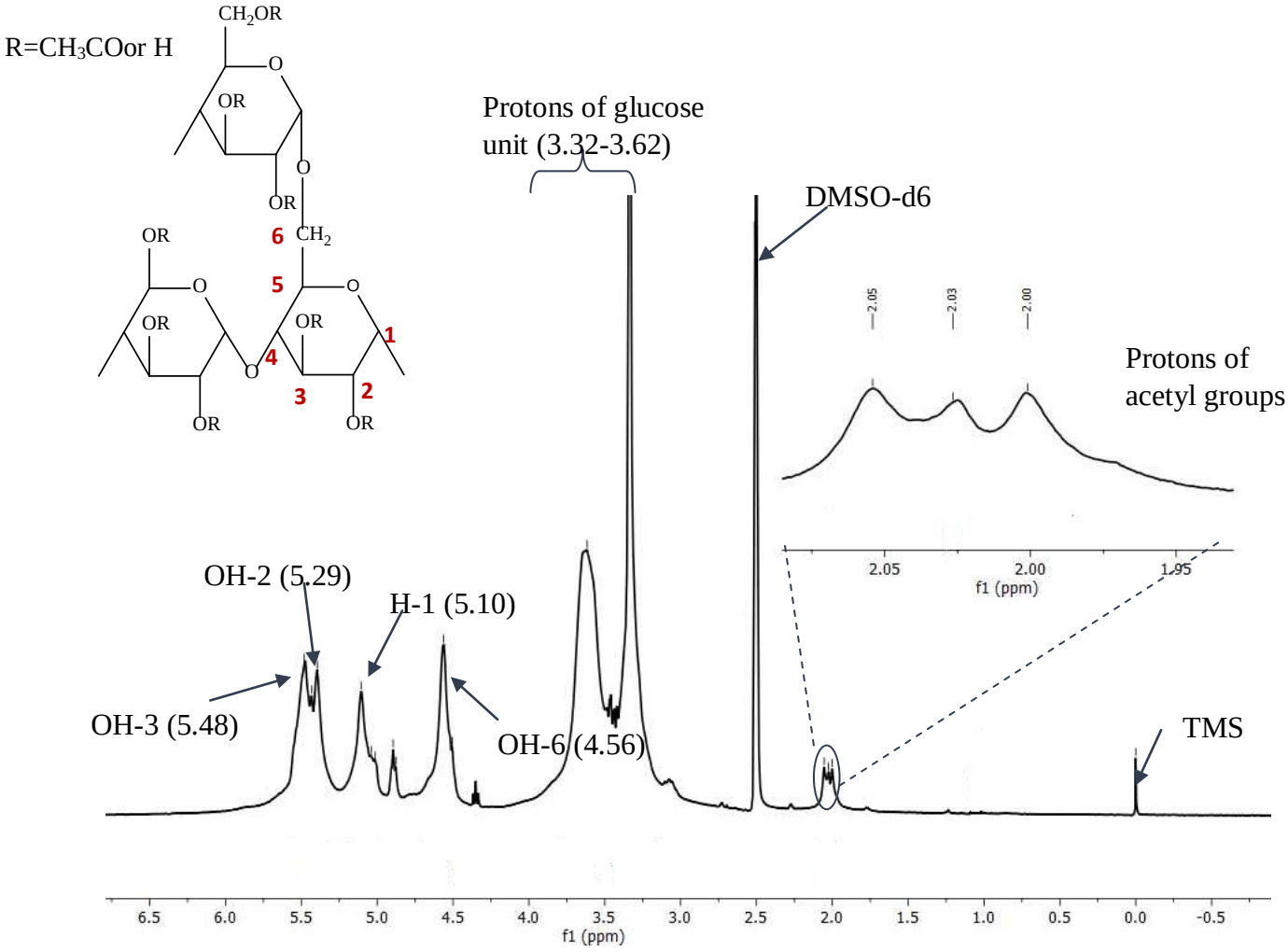



**Figure 4.5** Chemical shift assignment of $^{1}$H NMR spectrum of acetylated (20% acetic anhydride) waxy corn starch

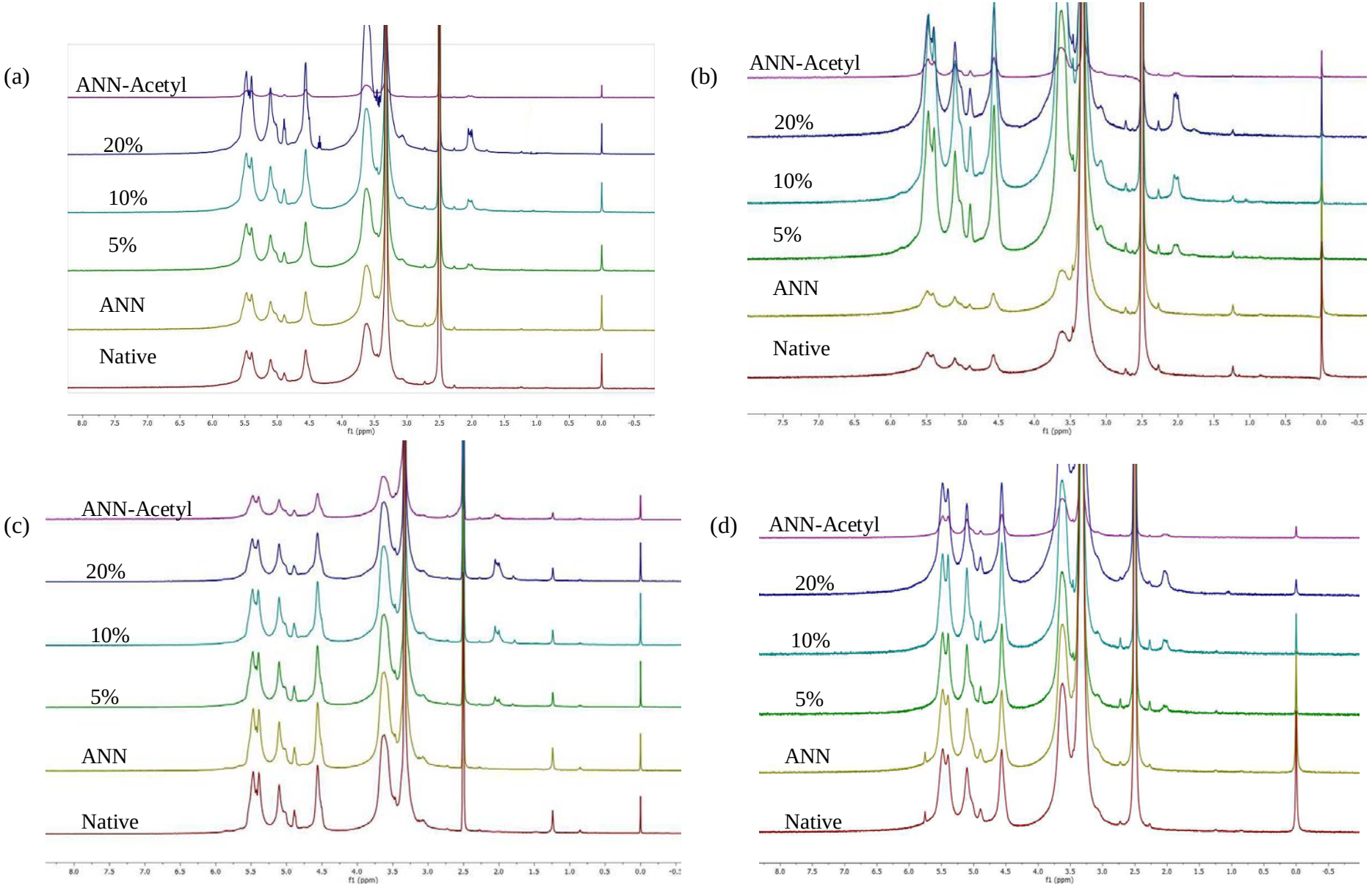



**Figure 4.6** $^{1}$H NMR spectra of native, annealed (ANN), acetylated (with 5, 10, and 20% acetic anhydride), and annealed-acetylated waxy corn (a), waxy rice (b), waxy barley (c), and waxy potato (d) starches.

### 4.7.3 X-ray diffraction patterns and crystallinity

The native WC, WB, and WR starches exhibited 'A-type' crystalline patterns, similar to that reported by Rocha et al. (2012), Waduge et al. (2006), and Wang et al. (2014). WP starch exhibited the 'B-type' crystalline pattern with reflection intensities centered at 5.5°, 17.2° and 22–24° 2θ. WC exhibited the highest relative crystallinity (42.4%), followed by WP> WR> WB. Differences in relative crystallinity among native starches can be attributed to differences in crystallite size, the amount of double helices that are arranged into a crystalline regions, average amylopectin chain length, orientation of the double helices (within the crystallites) to the X-ray beam, and starch moisture content (Cheetham & Tao, 1998).

Annealing did not change the crystalline pattern in waxy starches (Fig 4.7), which is in agreement with previous studies (Gomand et al., 2012; Rocha et al., 2012; Wang et al., 2014). Relative crystallinity of waxy corn increased upon annealing, while it remained unchanged in other waxy starches. Increase in relative crystallinity can be attributed to a better reorientation of double helices and/or increase in crystallite size (Lan et al., 2008; Rocha et al., 2012). However, in other waxy starches, the reorientation of double helices might have improved quality rather than increased quantity/size of starch crystallites (Wang et al., 2014).

Acetylation and dual modification did not alter the crystalline pattern in waxy starches which was in agreement with previous studies where mild acetylation was performed (Chang & Lv, 2017; Mbougueng et al., 2012; Singh et al., 2012). The acetylated waxy starches showed a slight decrease in the intensities of the peaks compared to their native counterparts. A significant decrease in relative crystallinity was observed with increasing DS in acetylated waxy starches. Similar results were reported in previous studies where acetylation reduced the crystallinity of low-amylose rice starch, and the lowest value of relative crystallinity was seen in acetylated

starch with the highest DS (DS 0.43-0.96) (Colussi et al., 2014). Acetylated waxy corn starch remained an A-type crystalline pattern and peaks at 15°, 17°, 18°, and 23° (2θ) were weakened as DS increased (DS from 0.3 to 1.08) (Luo & Shi, 2012). It is plausible that acetylation reduces the formation of intermolecular hydrogen bonds, resulting in a low ordered crystalline structure of starch granules (Luo & Shi, 2012). Even though annealing increased relative crystallinity in WC, and it did not change relative crystallinity in other waxy starches, dual modification showed a greater decrease in crystallinity, indicating that destruction of the starch structure caused by acetyl groups has negated the effect of crystalline perfection on annealing. Further, structural changes that occurred on annealing (as a result of increased amylopectin chain mobility in the amorphous lamellae and the movement of complete molecular sequences within a crystalline lattice) facilitated introduction of acetyl groups and the distribution of acetyl groups caused more destruction to the crystalline structure in dual modified waxy starches.

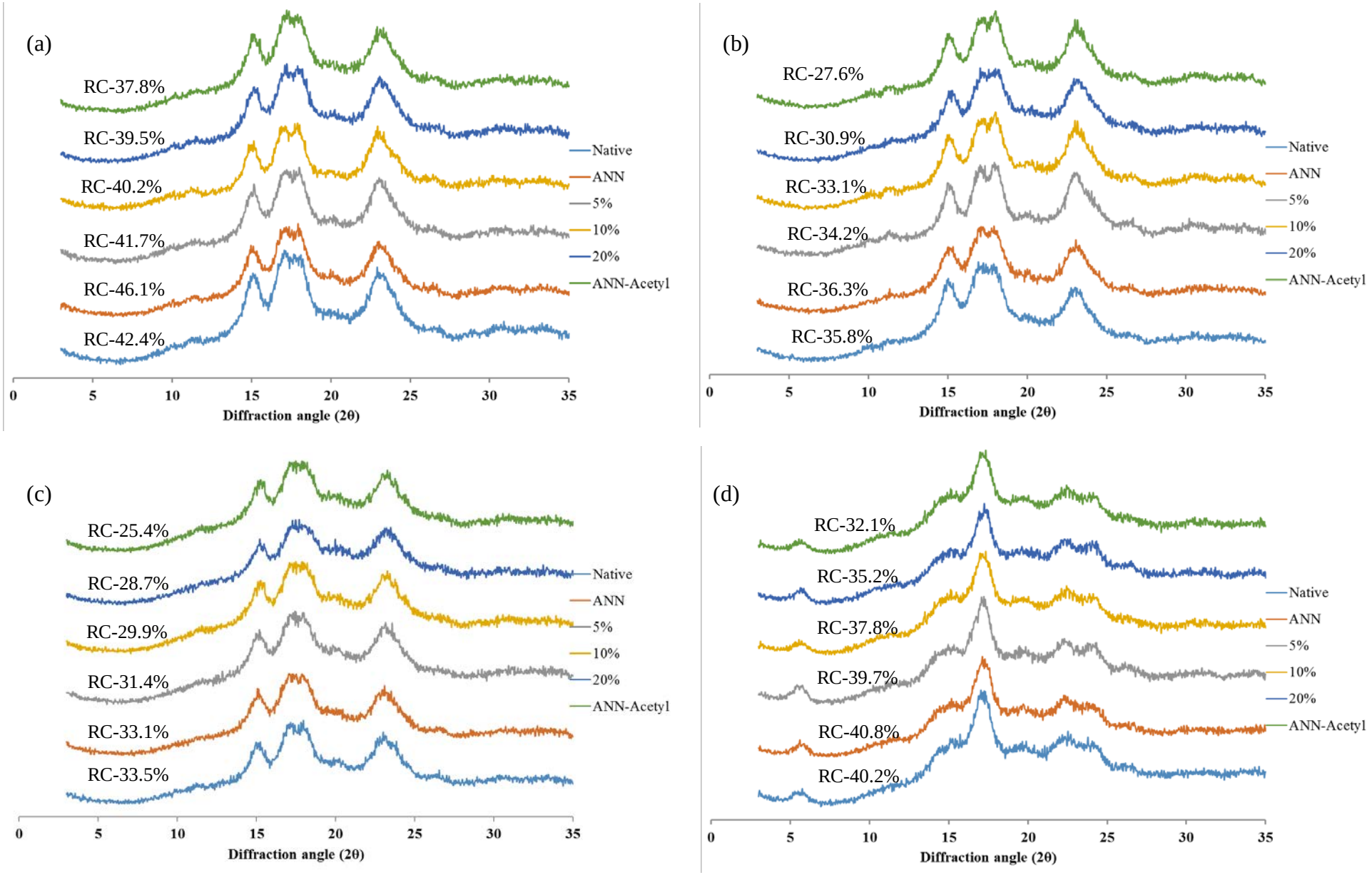



**Figure 4.7** X-ray diffraction patterns and relative crystallinity (RC) of native, annealed (ANN), acetylated (with 5, 10, and 20% acetic anhydride), and annealed-acetylated waxy corn (a), waxy rice (b), waxy barley (c), and waxy potato (d) starches.

## 4.8 Impact of annealing, acetylation, and annealing-acetylation on starch properties

### 4.8.1 Granule swelling

The swelling factor (SF) of native and modified waxy starches are presented in Table 4.4. Swelling factor increased with increasing temperature. In native starches, WP showed the highest SF followed by WB>WR>WC. The variations in SF in native starches can be attributed to the interplay between the extent of interaction of amylopectin chains and phosphorous content. Native WP starch had the highest phosphorus content (Table 4.1) and larger granules, which facilitated granule swelling. Phosphate monoesters, which have the properties of an anionic polyelectrolyte when they are dispersed into aqueous solutions and then repel one another, facilitate granule swelling (Vamadevan & Bertoft, 2015). In native waxy cereal starches, SF has shown relation to gelatinization transition temperatures, where the starch with highest gelatinization transition temperatures (WC) (Table 4.2) showed the lowest SF. Li and Yeh (2001) have shown that $T_p$ has a negative effect on granular swelling and high $T_p$ slowed the disruption of starch granule as well as the absorption of water by starch granule.

Annealing decreased SF throughout the temperature range. Reduction of SF upon annealing was also observed in waxy barley (Waduge et al., 2006), waxy rice (Zeng et al., 2015), and waxy wheat (Lan et al., 2008). The highest decrease was observed in waxy potato followed by WB>WR>WC. The reduction in SF upon annealing could be attributed to the interplay between crystalline perfection and interaction between amylopectin chains. WP showed the highest crystalline perfection upon annealing which was evident in DSC study (Table 4.2). Hence, it is plausible that the reduction in SF was highest in WP. In waxy cereal starches, WB had achieved more crystalline perfection, compared to WC and WR (Table 4.2), which can be related to a

greater decrease in SF of WB, especially at higher temperatures (85 and 90$^{o}$C) compared to WC and WR upon annealing. Increased crystalline perfection and starch chain interaction would decrease hydration of the amorphous region, hence decrease granule swelling (Lan et al., 2008).

Acetylation increased SF throughout the temperature range compared to native counterparts, and highest SF values were observed with higher DS in all the waxy starches at each temperature. Similarly, an increase in granule swelling upon acetylation was observed in wheat, potato, corn (Gunaratne & Corke, 2007), waxy, normal and high-amylose corn (Hong et al., 2018) and rice (Gonzalez & Pérez, 2002; Sodhi & Singh, 2005) starches. In acetylated WC, increase in SF from 60 to 70$^{o}$C was greater, but after 70$^{o}$C, the increase was only marginal and reached a plateau. Similarly SF reached a plateau in acetylated WR after 80$^{o}$C. However, in acetylated WB and WP starches, SF continuously increased with increasing temperature and acetylated WP showed the highest increased at the end of 90$^{o}$C. Native WP had a higher amount of phosphorous and weaker crystallites, and hence the introduction of acetyl groups could have caused crystallites weaker compared to other waxy starches causing a greater increase in granule swelling. In waxy cereal starches, WB had achieved more crystalline perfection upon ANN indicating that native WB had the weaker crystallites among waxy cereal starches, and during acetylation crystallites of WB starch could have weakened to a greater extent compared to acetylated WC and WR leading to a greater increase in SF. Acetylation weakens the bonding forces between starch chains and larger bulky acetyl groups prevent the inter-chain association causing an increase in the hydration and swelling of starch granules (Gunaratne & Corke, 2007). Sing et al. (2004) studied swelling power of acetylated potato and corn starches with various DS and found that swelling power increased with increasing DS in both starches and a greater increase was observed in potato starch.

Annealing followed by acetylation with 10% acetic anhydride caused an increase in SF in waxy cereal starches compared to native counterparts; however, the extent of increase varied with the starch source. In waxy cereal starches, SF values of dual modification were not significantly different ($p>0.05$) to that of acetylated starches with 5% acetic anhydride (lowest DS) throughout the temperature range except at 60$^{o}$C in WC. This showed that crystalline perfection on ANN had restricted the granule swelling to a considerable extent even though 10% of acetic anhydride was used during dual modification. Even though X-ray diffraction analysis resulted in a lower relative crystallinity (Fig 4.7) in dual modified waxy starches, a decrease in SF indicated that crystallites have become more stable upon the dual modification. In WP, at 60 and 70$^{o}$C, SF values of dual modification were not significantly different ($p>0.05$) from that of annealed WP, indicating that crystalline perfection on ANN would have overcome the weakening of bonds by acetylation at 60 and 70$^{o}$C. Dual modified WP showed SF values which lower than 5% acetic anhydride treatment but greater than native WP, above 80$^{o}$C. Since WP had achieved greater crystalline perfection on ANN (Table 4.2), it is plausible that disruption of granule structure during the dual modification would have less impact on WP, leading to restricted granule swelling compared to waxy cereal starches.

**Table 4.4** Swelling factor of native, annealed, acetylated, and annealed-acetylated waxy starches in the temperature range 60–90°C[1*]

| Starch source | Temperature (°C) | | | | |
|---|---|---|---|---|---|
| | 60 | 70 | 80 | 85 | 90 |
| **Waxy corn** | | | | | |
| Native | 5.65±0.38$^{d}$ | 15.19±0.33$^{d}$ | 15.01±0.32$^{b}$ | 19.23±0.89$^{c}$ | 19.21±0.27$^{d}$ |
| ANN | 3.75±0.00$^{e}$ | 13.24±0.79$^{e}$ | 12.59±0.16$^{b}$ | 17.63±0.18$^{d}$ | 17.18±0.71$^{e}$ |
| 5% | 11.94±0.75$^{c}$ | 35.18±0.29$^{c}$ | 35.37±0.93$^{a}$ | 34.73±0.47$^{b}$ | 34.67±0.59$^{c}$ |
| 10% | 16.08±0.74$^{b}$ | 39.05±0.16$^{b}$ | 38.09±0.93$^{a}$ | 37.57±0.40$^{a}$ | 37.21±0.47$^{ab}$ |
| 20% | 18.64±0.52$^{a}$ | 40.52±0.63$^{a}$ | 38.98±0.68$^{a}$ | 37.67±0.59$^{a}$ | 37.81±0.68$^{a}$ |
| ANN-Acetyl | 6.87±0.96$^{d}$ | 34.89±0.29$^{c}$ | 36.19±0.46$^{a}$ | 36.10±0.26$^{b}$ | 35.60±0.95$^{bc}$ |
| **Waxy rice** | | | | | |
| Native | 10.97±0.40$^{d}$ | 21.09±0.26$^{b}$ | 20.72±0.60$^{d}$ | 19.24±0.55$^{c}$ | 18.99±0.57$^{c}$ |
| ANN | 9.80±0.21$^{d}$ | 17.28±0.28$^{c}$ | 17.35±0.80$^{e}$ | 16.08±0.44$^{d}$ | 16.12±0.28$^{d}$ |
| 5% | 18.63±0.91$^{c}$ | 21.76±0.95$^{b}$ | 29.40±0.83$^{bc}$ | 28.57±0.50$^{b}$ | 27.97±0.45$^{b}$ |
| 10% | 22.73±0.32$^{b}$ | 23.27±0.96$^{b}$ | 30.94±0.39$^{ab}$ | 28.47±0.40$^{b}$ | 28.14±0.91$^{b}$ |
| 20% | 26.08±0.18$^{a}$ | 27.03±0.74$^{a}$ | 32.60±0.89$^{a}$ | 31.60±0.36$^{a}$ | 30.83±0.96$^{a}$ |
| ANN-Acetyl | 18.63±0.98$^{c}$ | 20.86±0.35$^{bc}$ | 28.09±0.91$^{c}$ | 27.80±0.40$^{b}$ | 26.75±0.76$^{b}$ |
| **Waxy barley** | | | | | |
| Native | 16.36±0.82$^{c}$ | 22.05±0.40$^{bc}$ | 25.61±0.36$^{bc}$ | 31.68±0.60$^{a}$ | 31.01±0.45$^{bc}$ |
| ANN | 11.09±0.00$^{d}$ | 20.47±0.31$^{c}$ | 23.61±0.14$^{c}$ | 27.21±0.24$^{c}$ | 26.16±0.14$^{d}$ |
| 5% | 22.52±0.75$^{b}$ | 22.65±0.96$^{ab}$ | 25.00±0.94$^{bc}$ | 27.77±0.25$^{bc}$ | 29.73±0.29$^{c}$ |
| 10% | 24.65±0.73$^{ab}$ | 23.23±0.72$^{ab}$ | 26.39±0.54$^{ab}$ | 28.67±0.38$^{b}$ | 31.94±0.92$^{b}$ |
| 20% | 24.14±0.86$^{ab}$ | 23.91±0.52$^{a}$ | 28.62±0.40$^{a}$ | 31.94±0.46$^{a}$ | 34.00±0.81$^{a}$ |
| ANN-Acetyl | 22.74±0.92$^{b}$ | 22.19±0.20$^{b}$ | 25.02±0.74$^{bc}$ | 27.53±0.45$^{bc}$ | 29.48±0.29$^{c}$ |
| **Waxy potato** | | | | | |
| Native | 29.38±0.70$^{c}$ | 39.50±0.30$^{c}$ | 39.21±0.22$^{e}$ | 39.43±0.12$^{e}$ | 37.57±0.31$^{e}$ |
| ANN | 19.12±0.36$^{d}$ | 31.72±0.34$^{d}$ | 32.94±0.21$^{f}$ | 32.79±0.26$^{f}$ | 31.82±0.13$^{f}$ |
| 5% | 30.32±0.67$^{c}$ | 41.01±0.97$^{bc}$ | 46.89±0.59$^{c}$ | 51.40±0.53$^{c}$ | 58.09±0.46$^{c}$ |
| 10% | 33.24±0.47$^{b}$ | 43.13±0.62$^{ab}$ | 49.45±0.92$^{b}$ | 52.87±0.50$^{b}$ | 60.51±0.71$^{b}$ |
| 20% | 44.63±0.98$^{a}$ | 45.30±0.36$^{a}$ | 56.35±0.82$^{a}$ | 61.00±0.50$^{a}$ | 65.07±0.39$^{a}$ |
| ANN-Acetyl | 20.68±0.93$^{d}$ | 32.66±0.45$^{d}$ | 41.58±0.44$^{d}$ | 42.83±0.47$^{d}$ | 46.85±0.56$^{d}$ |

[1] All data represent the mean of triplicates. Values followed by the same superscript in each column for each starch source are not significantly different ($p>0.05$).

[*]5, 10, and 20% represent acetylated starch with 5, 10, and 20% acetic anhydride, respectively.

### 4.8.2 Gelatinization

The gelatinization transition temperatures (onset [$T_o$], midpoint [$T_p$] and conclusion [$T_c$]), gelatinization transition temperature range ($T_c$-$T_o$) as well as the enthalpy of gelatinization ($\Delta H$) of native and modified are presented in Table 4.5. The extent of crystalline perfection was reflected in the gelatinization temperatures (Tester, 1997). In native waxy cereal starches, the highest gelatinization temperatures were observed in WC, followed by WR and WB, indicating that crystallites were more perfect in WC compared to WR and WB. Gelatinization temperatures of native WP starch were greater than WR and WB, but lower than WC. However, it was difficult to interpret whether WP starch had more perfect crystallites than WR and WB since WP has B-type crystalline structure and more phosphorous compared to A-type crystalline structure in waxy cereal starches and low amount of phosphorous.

Among the native starches, $T_o$, $T_p$, $T_c$ followed the order of WC>WP>WR>WB which is in accordance with the order of relative crystallinity (Fig 4.7). The degree of crystallinity plays an important role in gelatinization parameters (Singh et al., 2003) since starches with higher crystallinity require more thermal energy to melt the crystallites, resulting in higher gelatinization transition temperatures. Enthalpy of gelatinization represents unraveling of double helices in both crystalline and non-crystalline arrays. Highest $\Delta H$ was obtained in WP which can be due to longer chain length (Table 4.1), followed by WC>WB>WR. Higher melting enthalpy in waxy potato starch was also observed compared to waxy corn, barley, and wheat starches (Genkina, Wikman, Bertoft, & Yuryev, 2007).

In all starches, $T_o$, $T_p$ and $T_c$ increased, and $T_c$-$T_o$ decreased upon annealing. These changes have been attributed to perfection of crystalline structure of starch (Hoover & Vasanthan, 1993; Jacobs & Delcour, 1998; Lan et al., 2008; Tester et al., 1998, 2000; Waduge et al., 2006). The

increases in $T_o$, $T_p$, and $T_c$ upon annealing were highest in WP followed by WB>WC~WR. The results suggest that crystallites in native WP are less perfect than in other waxy starches, hence the impact of annealing was more pronounced in WP. Higher phosphorous content in waxy potato starch (Table 4.1) could be attributed to less perfect crystallites. Muhrbeck and Eliasson (1991) have shown that part of the phosphate groups interferes with the formation of ordered structure in potato starch. Similar results were reported by Muhrbeck and Svensson (1996), where the rates of the shifts in ΔH and peak temperature were more pronounced for the high phosphorylated potato starches. Annealing increases the mobility of amylopectin chains which causes reorientation of phosphate groups, and allow more amylopectin groups to interact, thus forming more ordered structure (Muhrbeck & Svensson,1996). Annealing did not change the gelatinization enthalpy in waxy starches indicating that new double helices were not formed on annealing. Similar results were observed for waxy barley (Waduge et al.,2006), waxy wheat (Lan et al., 2008), waxy corn (Wang et al., 2014) and waxy potato (Gomandet al., 2012) starches.

Acetylation caused decreases in gelatinization transition temperatures and enthalpy in all the waxy starches, and the decrease was greater with increasing DS. Similar results were observed in rice (Colussi et al., 2015), corn and potato (Sing et al., 2004), and hulless barley (Chang & Lv, 2017) starches, where the starch treated with the highest concentration of acetic anhydride had the lowest gelatinization temperatures and enthalpy. Substituted acetyl groups inhibit the inter-chain association of starch chains promoting granule hydration and extensively hydrated granules required less energy to reach gelatinization (Gunaratne & Corke, 2007). The decreases in $T_o$ after treatment with 20% acetic anhydride were 2.63, 4.59, 5.87, and 10.71 °C for WC, WR, WB, and WP, respectively. In waxy cereal starches, the decrease in $T_o$ after treatment with 20% acetic anhydride was in accordance with the DS, where a greater decrease was observed in

WB with DS of 0.22 followed by WR (DS 0.19) and WC (DS 0.09). However, WP which had DS of 0.07 after treatment with 20% acetic anhydride showed the highest decrease in $T_o$ indicating that introduction of acetyl groups along with the pre-existing phosphate groups have weakened the crystalline structure to a greater extent in WP.

However, the change in $T_c$-$T_o$ was only marginal in acetylated waxy starches indicating that even though acetylation distrupt the granule structure, it has not affected the homogeneity of the crystallites to a significant extent. Gelatinization enthalpy decreased with acetylation treatment, and at 20% acetic anhydride treatment, the decrease in ΔH compared to native starches was 4.9, 4.3, 1.95, and 4.98 J/g for WC, WR, WB, and WP, respectively. However, the decrease in ΔH did not follow the same order of decrease in $T_o$. The decrease in ΔH shows the disruption of crystalline structures in acetylated waxy starches which was evident in relative crystallinity observed in X-ray diffraction study (Fig 4.7), where the relative crystallinity decreased with increasing DS of acetylated starches.

Gelatinization transition temperatures of dual modified waxy starches were higher than that of acetylated starches and were not significantly different ($p>0.05$) from the gelatinization temperatures of annealed starches except for $T_o$ in WR and WB. However, ΔH of annealed acetylated starches were siginificantly lower ($p<0.05$) than native, annealed, and acetylated starches. Since gelatinization transition temperatures represent crystal stability while ΔH reflects the melting enthalpy of both packing crystal and amylopectin based double helices (Chang & Lv, 2017), it could be explained that crystalline perfection caused by annealing made the crystallites more stable, but it was not sufficient to prevent disruption of packing of crystals during the dual modification. Further, dual modified starches had the lowest relative crystallinity (Fig 4.7) indicating the greater damage to the packing of crystallites during the treatment. It could be

plausible since annealing increases the starch chain mobility which provides more access to acetyl groups and the distribution of acetyl groups in the starch chains during dual modification might have caused more damage to the packing of crystals than in acetylated starches. Furthermore, dual modification significantly decreased ($p<0.05$) $T_c$-$T_o$, comapred to native and acetyalted counterparts indicating increased homogeneity of the crystallites.

**Table 4.5** Gelatinization parameters of native, annealed, acetylated, and annealed-acetylated waxy starches[1*]

| Starch source | Gelatinization transition parameters[2] | | | | |
|---|---|---|---|---|---|
| | $T_o$ (°C)[3] | $T_p$ (°C)[3] | $T_c$ (°C)[3] | $T_c$-$T_o$ (°C)[4] | ΔH (J/g)[5] |
| **Waxy corn** | | | | | |
| Native | 64.22±0.04$^{c}$ | 70.34±0.25$^{b}$ | 76.33±0.06$^{b}$ | 12.11±0.01$^{b}$ | 14.85±0.23$^{a}$ |
| ANN | 69.92±0.01$^{a}$ | 73.67±0.01$^{a}$ | 78.27±0.10$^{a}$ | 8.35±0.08$^{d}$ | 14.62±0.36$^{a}$ |
| 5% | 63.17±0.16$^{d}$ | 69.43±0.37$^{bc}$ | 75.78±0.10$^{bc}$ | 12.62±0.10$^{a}$ | 11.76±0.18$^{b}$ |
| 10% | 62.65±0.10$^{d}$ | 68.54±0.52$^{cd}$ | 75.35±0.21$^{c}$ | 12.79±0.10$^{a}$ | 10.07±0.30$^{c}$ |
| 20% | 61.59±0.37$^{e}$ | 67.27±0.37$^{d}$ | 74.22±0.39$^{d}$ | 12.63±0.00$^{a}$ | 9.95±0.33$^{c}$ |
| ANN-Acetyl | 68.61±0.40$^{b}$ | 73.25±0.40$^{a}$ | 78.75±0.40$^{a}$ | 10.15±0.20$^{c}$ | 6.87±0.66$^{d}$ |
| **Waxy rice** | | | | | |
| Native | 59.07±0.18$^{c}$ | 66.50±0.23$^{b}$ | 72.84±0.25$^{b}$ | 13.77±0.42$^{b}$ | 11.29±0.09$^{a}$ |
| ANN | 65.52±0.44$^{a}$ | 69.84±0.45$^{a}$ | 74.59±0.52$^{a}$ | 9.07±0.08$^{c}$ | 11.67±0.05$^{a}$ |
| 5% | 58.83±0.03$^{d}$ | 66.04±0.01$^{b}$ | 72.70±0.02$^{b}$ | 13.87±0.01$^{ab}$ | 10.01±0.05$^{b}$ |
| 10% | 58.04±0.15$^{d}$ | 65.86±0.27$^{b}$ | 72.62±0.01$^{b}$ | 14.59±0.13$^{a}$ | 9.01±0.04$^{b}$ |
| 20% | 54.54±0.04$^{e}$ | 62.04±0.00$^{c}$ | 69.07±0.11$^{c}$ | 14.53±0.15$^{ab}$ | 6.99±0.17$^{c}$ |
| ANN-Acetyl | 64.18±0.06$^{b}$ | 69.29±0.04$^{a}$ | 73.92±0.08$^{a}$ | 9.74±0.03$^{c}$ | 5.34±0.59$^{d}$ |
| **Waxy barley** | | | | | |
| Native | 56.90±0.06$^{c}$ | 61.40±0.00$^{b}$ | 67.10±0.05$^{b}$ | 10.20±0.11$^{ab}$ | 11.90±0.13$^{a}$ |
| ANN | 64.56±0.04$^{a}$ | 66.74±0.11$^{a}$ | 69.95±0.17$^{a}$ | 5.40±0.13$^{d}$ | 12.02±0.21$^{a}$ |
| 5% | 55.45±0.28$^{d}$ | 60.08±0.08$^{c}$ | 65.44±0.09$^{c}$ | 9.99±0.37$^{b}$ | 11.76±0.18$^{a}$ |
| 10% | 52.75±0.24$^{e}$ | 57.91±0.32$^{d}$ | 63.63±0.28$^{d}$ | 10.88±0.04$^{a}$ | 10.07±0.30$^{b}$ |
| 20% | 51.03±0.05$^{f}$ | 56.39±0.14$^{e}$ | 61.46±0.21$^{e}$ | 10.44±0.26$^{ab}$ | 9.95±0.33$^{b}$ |
| Ann-Acetyl | 62.87±0.01$^{b}$ | 66.19±0.16$^{a}$ | 70.26±0.11$^{a}$ | 7.39±0.11$^{c}$ | 5.15±0.21$^{c}$ |

**Table 4.5** *(continued)*

| Starch source | Gelatinization transition parameters[2] | | | | |
|---|---|---|---|---|---|
| | $T_o$ ($^oC$)[3] | $T_p$ ($^oC$)[3] | $T_c$ ($^oC$)[3] | $T_c$-$T_o$ ($^oC$)[4] | $\Delta H$ (J/g)[5] |
| **Waxy potato** | | | | | |
| Native | 62.92±0.47$^b$ | 68.32±0.49$^b$ | 73.64±0.24$^b$ | 11.05±0.23$^{bc}$ | 17.51±0.06$^a$ |
| ANN | 71.01±0.04$^a$ | 73.94±0.02$^a$ | 78.30±0.03$^a$ | 7.29±0.07$^e$ | 17.84±0.26$^a$ |
| 5% | 62.82±0.03$^b$ | 67.95±0.15$^b$ | 73.42±0.39$^b$ | 10.60±0.42$^c$ | 14.01±0.34$^b$ |
| 10% | 61.24±0.57$^c$ | 67.09±0.63$^b$ | 72.93±0.39$^b$ | 11.69±0.18$^{ab}$ | 13.21±0.16$^b$ |
| 20% | 59.21±0.12$^d$ | 65.61±0.12$^c$ | 71.07±0.08$^c$ | 11.87±0.04$^a$ | 12.53±0.09$^b$ |
| ANN-Acetyl | 70.35±0.04$^a$ | 74.09±0.19$^a$ | 78.81±0.13$^a$ | 8.46±0.17$^d$ | 7.36±0.99$^c$ |

[1]All data represent the mean of triplicates. Values followed by the same superscript in each column in each starch source are not significantly different ($p>0.05$).

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

[3]$T_o$,$T_p$, and $T_c$ indicate the onset, peak, and end temperature of gelatinization, respectively.

[4]($T_c$-$T_o$) indicates the gelatinization temperature range.

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

[*]5, 10, and 20% represent acetylated starch with 5, 10, and 20% acetic anhydride, respectively.

### 4.8.3 Enzyme hydrolysis

Enzyme hydrolysis of native and modified starches by a mixture of pancreatin and amyloglucosidase is given in Fig 4.8. Progressive increases in the percentage of hydrolysis were observed in native and modified waxy starches. In native waxy starches, WR showed the highest enzyme susceptibility followed by WC~WB>WP. All waxy cereal starches were completely hydrolyzed at the end of 24 hours, while the percentage hydrolysis of WP was 28.7%. Amylase hydrolysis involves an enzyme in solution acting on a solid starch substrate, and hence, the accessible surface area and the adsorption efficiency of enzyme are important kinetic parameters (Bertoft & Manelius, 1992). Therefore, granule size plays an important role in enzyme susceptibility, where smaller granules hydrolyze to a greater extent than the larger granules due to relatively larger surface area per unit mass in smaller granules, which may increase the extent of enzyme binding and ultimately result in greater hydrolysis (Naguleswaran, Vasanthan, Hoover & Bressler, 2013). This was evident as native WR which had the smallest granules (Fig. 4.1b) showed the highest hydrolysis rate, while native WP which had the largest granules (Fig. 4.1d) showed the lowest hydrolysis rate. WP has a B-type crystalline structure which is less susceptible to enzyme hydrolysis compared to A-type crystalline structure in waxy cereal starches (Fig. 4.7), since A-type starches have more short chains (DP 6-12) and branch points which are scattered in crystalline regions, creating weak points and making more susceptible to enzymatic hydrolysis (Jane et al., 1997). Both WC and WB starches have surface pores (Fig. 4.1a and c), which provide access for initial enzyme attack that causes the enzyme to penetrate to the granule interior (Sujka & Jamroz, 2007).

Annealing increased the enzyme susceptibility in WC and WB, while decreased in WR and WP. The size and number of pores might have increased in WC and WB upon annealing, making the

starch granules more accessible to enzymes, even though changes in pore size/number was not observed during SEM. Slight increase in pore size on annealing in waxy barley (Waduge et al., 2006) and presence of more pores on the surface of some annealed waxy corn granules (Rocha et al., 2012) was reported, indicating that changes in granule pores upon annealing may have contributed to the increased susceptibility towards enzyme attack. Rocha et al. (2012) observed increased susceptibility to enzyme hydrolysis of corn starch upon annealing and suggested that the presence of more surface pores in some annealed granules, as a result of the action of endogenous amylases during annealing, may have contributed to greater exposure of the starch granules to the exogenous enzymes. The decrease in enzyme hydrolysis on annealing in WR and WP can be attributed to the crystalline perfection and increased interaction between starch chains which limit the accessibility of enzymes. Both native and annealed WR almost completely hydrolyzed after 16h, but WP showed limited hydrolysis even after 72h, and the decrease in hydrolysis upon annealing was more evident in WP at the later stages (after 8h) of hydrolysis. The significant decrease in enzyme susceptibility of WP can be attributed to the greater crystalline perfection achieved upon annealing (Table 4.5) which retard the enzyme attack.

Acetylation decrease the susceptibility towards enzyme hydrolysis in waxy starches, where the decrease was greater with increasing acetic anhydride concentration/DS. It has been suggested that the introduction of chemical groups restricts α-amylase attack on adjacent α-D-(1→4) glycosidic linkages (Hoover & Sosulski, 1985). However, the extent of decrease was not consistent throughout the hydrolysis period and in different waxy starches. At the end of 72h period, hydrolysis % of acetylated starches with 20% acetic anhydride was 73.87, 71.07, 62.10, and 26.06% in WC, WR, WB, and WP, respectively. In waxy cereal starches, hydrolysis % could be related to the DS, where WB which had the highest DS (0.22) showed the lowest hydrolysis

% at the end of 72h. Colussi et al. (2015) studied the susceptibility of acetylated rice starch towards α-amylase hydrolysis and found that the different levels of acetylation did not significantly change the hydrolysis of starch. Chung et al. (2008) reported that digestibility of acetylated corn starch towards pancreatic α-amylase decreased at the later stage of hydrolysis (after 3 h).

Dual modification decreased the % hydrolysis compared to native and annealed waxy starches, but the extent of decreased varied. In WC starch annealed-acetylated treatment (DS 0.13) showed the lowest hydrolysis throughout the hydrolysis period, whereas dual modified WP (DS 0.12) starch showed the lowest hydrolysis after 8 h period. In dual modified WR starch (DS 0.25) the extent of hydrolysis was not significantly different ($p>0.05$) from that of acetylated WR starch with 10% acetic anhydride (DS 0.05) during the first 16 h of hydrolysis. A similar trend was observed in dual modified WB starch (DS 0.24), where the % hydrolysis was not significantly different ($p>0.05$) from that of acetylated WB with 10% acetic anhydride (DS 0.05) except 0.5, 8, and 16 h period. Dual modified waxy starches showed significantly lower ($p<0.05$) hydrolysis % compared to annealed starches and except for WB and early stage of hydrolysis in WR (first 16h) and WP (first 4 h), dual modified waxy starches showed the lowest hydrolysis %, indicating that distribution of acetyl groups in the starch chains during dual modification causes greater steric hindrance to enzyme attack.

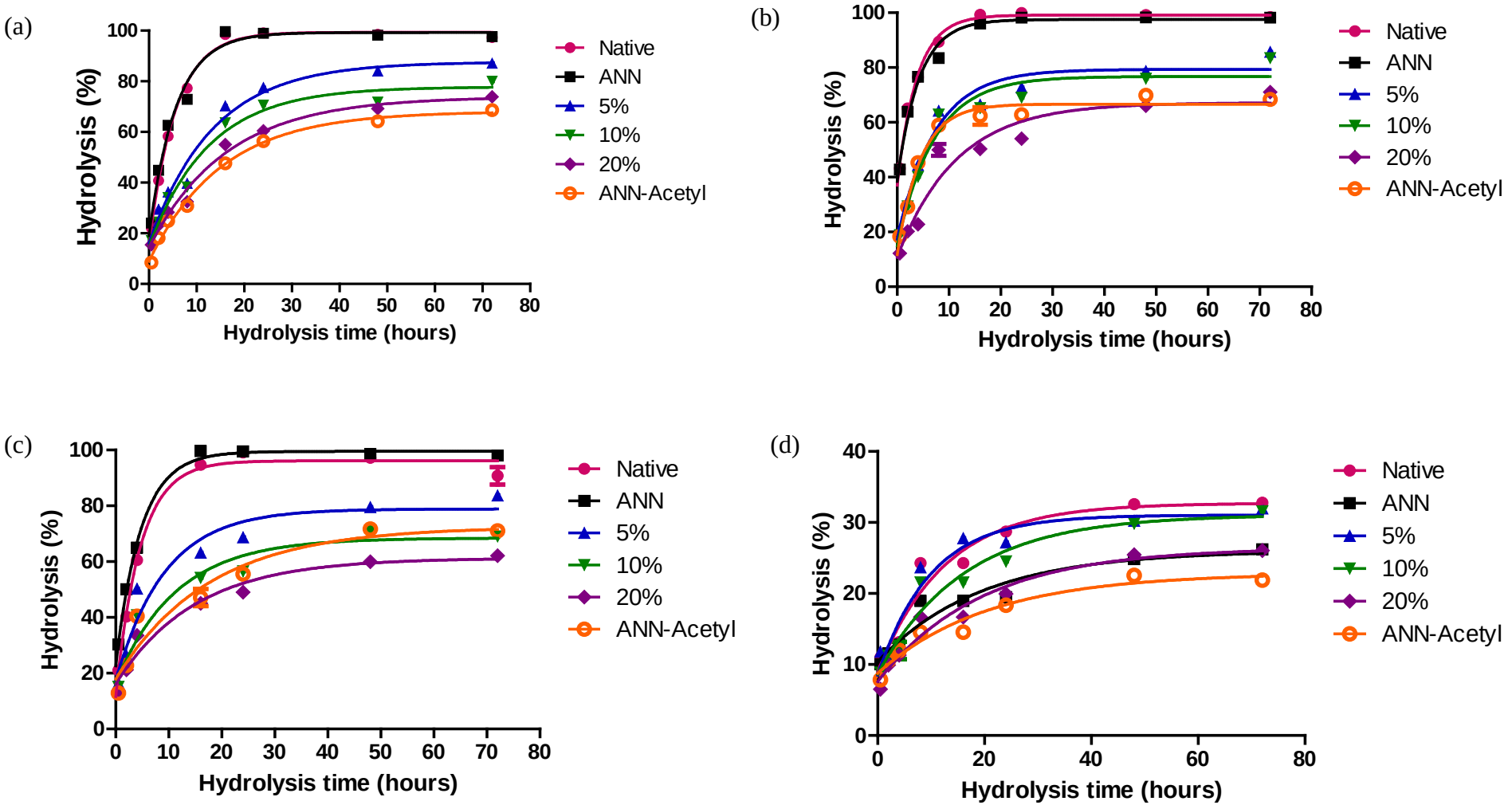



**Figure 4.8** Digestibility profiles of native, annealed, acetylated (with 5, 10, and 20% acetic anhydride), and annealed-acetylated waxy corn (a), waxy rice (b), waxy barley (c), and waxy potato (d) starches.

#### 4.8.3.1 Starch nutrition fractions

Rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) of native and modified waxy starches are presented in Table 4.6. In native starches, WR had the highest RDS content followed by WC~WB>WP as a result of greater hydrolysis rate of WR starch. SDS contents were significantly higher in waxy cereal starches (56.05-76.46%) compared to WP starch (13.30%). Native cereal starches are known to contain a larger portion of SDS since their A-type crystalline structure is more susceptible to enzymatic hydrolysis compared to the B-type starches (Zhang & Hamaker, 2009). RS contents of native waxy cereal starches (0.73-5.32%) were lower than that of WP starch which had RS content of 75.73%. RDS and SDS levels were much higher, and RS level was much lower than reported values of native WC (Wang et al., 2014) and WR (Zeng et al., 2015). In a previous study, a similar RS content was reported for WP starch, while RDS content was higher and SDS content was lower (Xie, Hu, Jin, Xu, & Chen, 2014) than the values obtained for WP in the current study. Differences in enzymatic hydrolysis of starch are influenced by a variety of factors which greatly affect the rate and efficiency of hydrolysis (Colussi et al., 2015). These factors include botanical origin, the concentration of the starch solution, type of enzyme, enzyme concentration, thermal stability, pH and the temperature of the reaction, and morphological and ultra-structural features of starch (Li, Vasanthan, Hoover, & Rossnagel, 2004; Foresti, del Pilar Williams, Martínez-García, & Vázquez, 2014).

Waxy cereal starches did not show significant differences in nutrition fraction upon annealing, except for an increase in RDS in WC and WB. However, annealed WP had significantly lower ($p<0.05$) SDS and significantly higher ($p<0.05$) RS contents compared to the native counterpart. A decrease in RDS contents and increase in SDS and RS levels upon annealing were observed in

waxy rice (Hung et al., 2016). However, Zeng et al. (2015) reported an increase in RDS contents and a decrease in SDS and RS contents of waxy rice upon annealing. Annealing treatment altered the enzymatic hydrolysis of the waxy corn starch, but the changes were small and insignificant (Wang et al., 2014). Changes to the presence of pores upon annealing in WC and WB starches might have affected the increase in RDS contents. Even though annealing did not change the RDS content in WP, increase in RS content, and a decrease in SDS contents showed that structural changes occurred on annealing had restricted the enzyme attack on annealed WP. Annealing causes crystalline perfection and facilitates starch chain interactions, and hence, theoretically, annealing should decrease starch susceptibility towards enzyme attack and RDS contents and increase SDS and RS fractions. However, the variations in nutritional fractions upon annealing of waxy starches can be due to the interplay between changes to granule surface and crystalline perfection and starch chain interactions (Jaykody & Hoover, 2008).

Acetylation decreased RDS and SDS levels and increased RS contents in waxy starches compared to native and annealed counterparts, and highest RS contents were obtained with 20% acetic anhydride treatment. The decreases in RDS contents were 6.5, 31.07, 7.38, and 4.45 and the increases in RS contents were 43.42, 47.87, 49.60, and 7.58 with 20% acetic anhydride treatment in WC, WR, WB, and WP, respectively. Acetylation treatment showed a significant impact on RDS content of WR compared to other waxy starches, showing that even with the greater surface area of WR, acetyl groups can limit the enzyme accessibility. Acetylated waxy starches swelled to a greater extent than the native counterparts (Table 4.4), and acetylation has caused slight changes on the granule surface (Fig 4.2). However, the RDS contents in acetylated waxy starches were less than that of native starches indicating that the acetyl groups could hinder the enzymatic action even during the early stage of hydrolysis. Similar results were observed in

corn (Chung et al., 2008), hulless barley (Chang & Lv, 2017) and rice (Xu et al., 2012) starches, where acetylation reduced RDS and SDS levels and increased RS content. However, acetylation treatment decreased RDS and increased SDS and RS in waxy corn (Hong et al., 2018). Furthermore, a relationship between DS and RS levels was not found in hulless barley (Chang & Lv, 2017) and rice (Xu et al., 2012) starches. Introduction of acetyl groups could change the spatial structure of starch molecules altering the binding sites between α-amylase and starch molecule resulting in high RS contents (Xu et al., 2012).

The dual modification resulted in a significant decrease ($p<0.05$) in RDS and SDS contents and a significant increase ($p<0.05$) in RS levels compared to native and annealed counterparts. Dual modification resulted in the highest RS content in WC. The RS content of dual modified WR was not significantly different ($p>0.05$) to that of acetylated starch with 5% acetic anhydride, while in WB and WP, RS contents were not significantly different ($p>0.05$) to that of acetylated starch with 20% acetic anhydride. It could be postulated that starch chain mobility occurred during annealing has facilitated the esterification process and the distribution of acetyl groups in dual modified starches could hinder the enzyme attack better than single acetylation treatment. The increased amylopectin chain mobility and movements of the starch crystallites on annealing might have created better spatial changes to facilitate the introduction of acetyl groups and the substitution distribution in dual modified starches might be different than single acetylation treatment which created better resistance to enzyme attack. Except for WR, acetylation (with 10% acetic anhydride) of annealed starches had increased the RS content which was similar to or higher than that of 20% acetic anhydride treatment indicating that dual modification is a potential technique to increase RS contents in waxy starches with less chemical usage.

**Table 4.6** Nutritional fractions of native, annealed, acetylated, and annealed-acetylated waxy starches determined by *in vitro* hydrolysis.[1*]

| Starch source | RDS (%)[2] | SDS (%) [2] | RS (%)[2] |
|---|---|---|---|
| **Waxy corn** | | | |
| Native | 22.01±0.09$^{b}$ | 76.46±0.47$^{a}$ | 1.53±0.47$^{e}$ |
| ANN | 24.06±0.56$^{a}$ | 75.60±0.09$^{a}$ | 0.34±0.66$^{e}$ |
| 5% | 17.35±0.09$^{c}$ | 52.92±0.38$^{b}$ | 29.73±0.28$^{d}$ |
| 10% | 17.15±0.19$^{cd}$ | 46.47±0.47$^{c}$ | 36.38±0.28$^{c}$ |
| 20% | 15.49±0.85$^{d}$ | 39.56±0.47$^{d}$ | 44.95±0.38$^{b}$ |
| ANN-Acetyl | 8.44±0.09$^{e}$ | 39.16±0.0.93$^{d}$ | 52.40±0.93$^{a}$ |
| **Waxy rice** | | | |
| Native | 43.22±0.19$^{a}$ | 56.05±0.09$^{a}$ | 0.73±0.28$^{c}$ |
| ANN | 42.75±0.47$^{a}$ | 53.32±0.38$^{a}$ | 3.39±0.09$^{c}$ |
| 5% | 20.81±0.28$^{b}$ | 45.61±0.56$^{b}$ | 33.58±0.85$^{b}$ |
| 10% | 18.88±0.56$^{c}$ | 45.21±0.19$^{b}$ | 35.91±0.38$^{b}$ |
| 20% | 12.17±0.28$^{d}$ | 39.23±0.94$^{c}$ | 48.60±0.66$^{a}$ |
| ANN-Acetyl | 18.35±0.38$^{c}$ | 44.01±0.97$^{bc}$ | 37.64±0.92$^{b}$ |
| **Waxy barley** | | | |
| Native | 21.01±0.00$^{b}$ | 73.67±0.38$^{a}$ | 5.32±0.38$^{d}$ |
| ANN | 30.32±0.56$^{a}$ | 69.28±0.38$^{a}$ | 0.40±0.19$^{d}$ |
| 5% | 16.49±0.56$^{c}$ | 46.74±0.94$^{b}$ | 36.77±0.85$^{c}$ |
| 10% | 15.16±0.38$^{c}$ | 39.03±0.94$^{c}$ | 45.81±0.93$^{b}$ |
| 20% | 13.63±0.09$^{d}$ | 31.45±0.93$^{d}$ | 54.92±0.97$^{a}$ |
| ANN-Acetyl | 12.90±0.19$^{d}$ | 34.24±0.97$^{cd}$ | 52.86±0.94$^{a}$ |
| **Waxy potato** | | | |
| Native | 10.97±0.09$^{a}$ | 13.30±0.56$^{a}$ | 75.73±0.47$^{d}$ |
| ANN | 10.04±0.28$^{b}$ | 8.91±0.75$^{cd}$ | 81.05±0.47$^{b}$ |
| 5% | 11.10±0.47$^{a}$ | 12.57±0.09$^{ab}$ | 76.33±0.38$^{dc}$ |
| 10% | 8.84±0.09$^{c}$ | 12.70±0.09$^{ab}$ | 78.46±0.00$^{c}$ |
| 20% | 6.52±0.00$^{e}$ | 10.17±0.47$^{bc}$ | 83.31±0.47$^{a}$ |
| ANN-Acetyl | 7.85±0.19$^{d}$ | 6.72±0.85$^{d}$ | 85.44±0.66$^{a}$ |

[1]All data represent the mean of triplicates. Values followed by the same superscript in each column in each starch source are not significantly different ($p>0.05$).

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

[*]5, 10, and 20% represent acetylated starch with 5, 10, and 20% acetic anhydride, respectively.

### 4.8.4 Acid hydrolysis

The hydroxonium ion ($H_3O^+$) from aqueous HCl randomly cleaves the glycosidic bond between glucose units in starch and thereby solubilize the starch. Studies demonstrate that acid hydrolysis shows a two-stage hydrolysis pattern, namely a fast initial rate followed by a slower subsequent rate (Robin et al., 1974; Jane et al., 1997; Hoover, 2000; Jayakody & Hoover, 2002). The initial fast initial rate is attributed to the hydrolysis of the amorphous regions within starch granules, whereas the later slow rate is attributed to the hydrolysis of crystalline regions (Jayakody & Hoover, 2002). It was difficult to identify a two-stage pattern in this study, even though a relatively slow rate was observed during the first three days, followed by a faster rate and reached a plateau after 30 days of hydrolysis (Fig. 4.9). Since the hydrolysis did not show a slow rate until 30$^{th}$ day, it is indicative that the crystalline regions of the tested starches were not hydrolyzed until 30$^{th}$ day. Similar hydrolysis pattern was observed for waxy wheat starch, where the slow rate was not observed even after 20 days of hydrolysis (Lan et al., 2008).

The native WB and WR starches showed greater hydrolysis rate followed by WP and WC during the first 7 days. However, this pattern varied during the period of 9$^{th}$ to 30$^{th}$ day. The extent of increase in hydrolysis between day 9 and 30 was higher in WC (39.8-81.9%), followed by WB (43.5-84.1%), WP (45.6-84.3%), and WR (44.0-80.9%). At the end of 35$^{th}$ day of hydrolysis, native WC, WB, WR, and WP starches were hydrolyzed to the extent of 82.7, 84.1, 80.9, and 83.8%, respectively. Difference in the extent of acid hydrolysis among starches has been attributed to the interplay of the following factors: (1) packing arrangement of starch chains in the amorphous and crystalline domains (Hoover, 2000), (2) pores on the granule surface (Jayakody & Hoover, 2002), and (3) granule size (Vasanthan & Bhatty, 1996), and (4) amylopectin chain length distribution (Raghunathan, Hoover, Waduge, Liu, & Warkentin, 2017).

Both WC and WB starches had surface pores (Fig. 4.1a and c), but WB showed a greater hydrolysis rate than WC, which can be due to the greater packing density of amylopectin chains (more perfect crystallites; Table 4.5), and lower proportion of short chains (DP 6-12) (Table 4.1) in WC than WB. Even though native WP had the lowest proportion of short chains (Table 4.1), and larger granule size (less surface area accessible to acid), the rate of hydrolysis was greater in WP compared to other waxy starches after several days of hydrolysis (day 9, 15, 25, and 30) indicating weaker packing arrangement of starch chains in WP. This was due to the presence of phosphate monoesters in the granule which counteracts the effect of the lower proportion of short chains and granule size on the rate and extent of acid hydrolysis.

The difference in the extent of hydrolysis between native and annealed starches was more pronounced in WP than in the other waxy starches. In native and annealed WC and WR starches, the difference was only marginal throughout the hydrolysis time. However, there was a significant decrease in the extent of hydrolysis in WB at the later stage (after 12 days). The decrease in hydrolysis in WP and WB upon annealing can be attributed to the crystalline perfection. This is plausible since the greater crystalline perfection (increase in gelatinization temperature) was achieved by WP followed by WB (Table 4.5). The crystalline perfection on annealing renders the chair to half chair conformation of glucose molecules, which occurs during acid hydrolysis (Hoover, 2000), thus increasing the resistance to acid attack.

Acid hydrolysis of acetylated and annealed-acetylated waxy starches was not performed since acid can hydrolyze the ester bonds resulting in the removal of acetyl groups from the modified waxystarches.

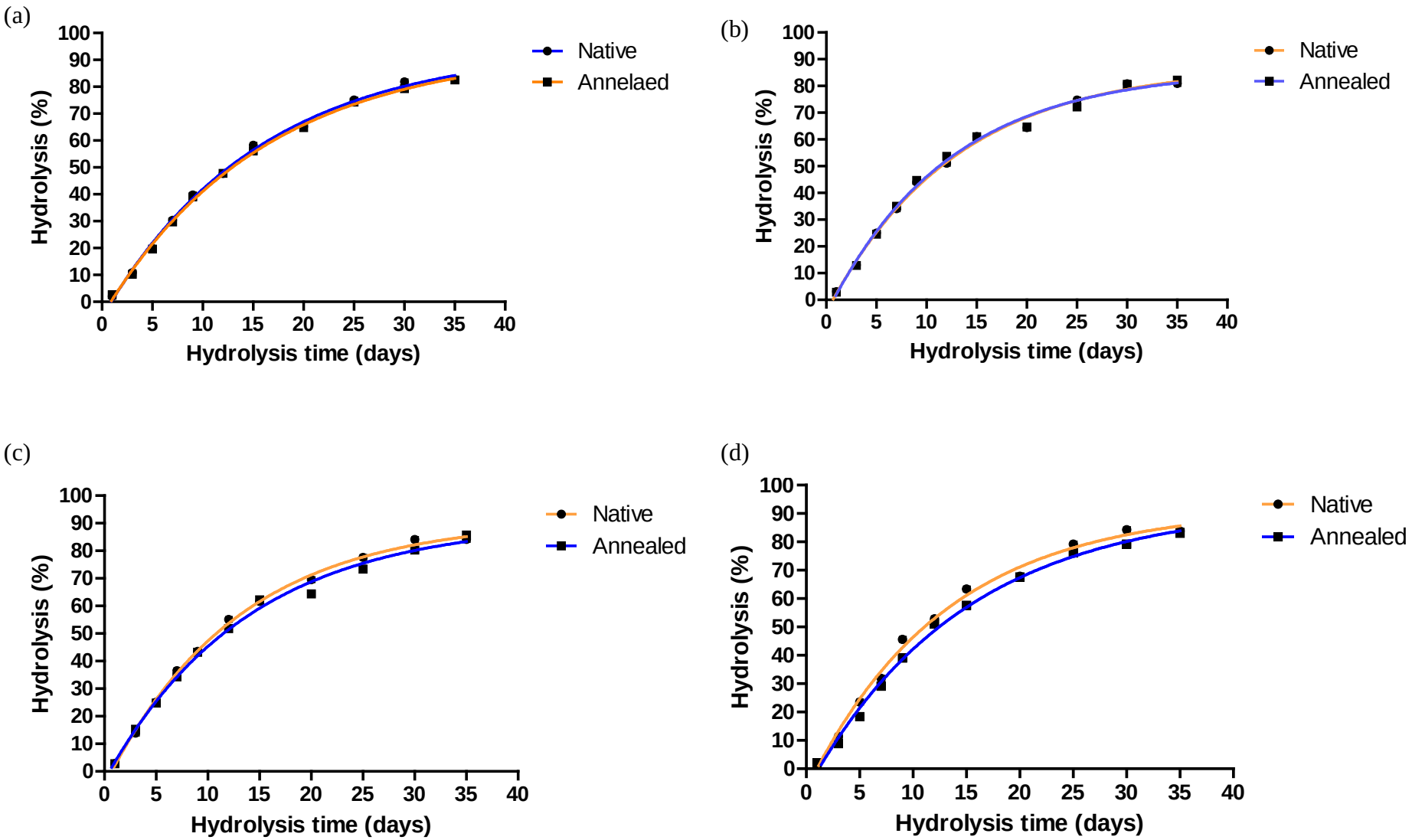



**Figure 4.9** Acid hydrolysis profiles of native and annealed waxy corn (a), waxy rice (b), waxy barley (c), and waxy potato (d) starches

### 4.8.5 Retrogradation

#### 4.8.5.1 Turbidity measurements

In a polymer–solvent system turbidity is caused by density fluctuations over the same distance scale and is most likely due to extensive polymer-polymer aggregation (Ambigaipalan et al., 2013). The formation of an amylose matrix gel is attributed to the initial gel firmness during retrogradation, which is completed within the first few hours of storage, followed by a slow increase in gel firmness that occurs over longer periods (days) due to reversible crystallization of amylopectin (Ring et al., 1987). Native WP starch showed higher light transmittance (%) than native cereal starches (Fig 4.10) which can be attributed to the presence of fewer granule remnants in the WP starch paste. Similar results were observed in native potato starch with higher light transmittance compared to corn starch, and it was suggested that potato starch granules are fragile during pasting and remnants of granules are largely absent from the paste giving higher light transmittance (Sing et al., 2004). In waxy cereal starches, WC showed the higher light transmittance followed by WB>WR, indicating that more granule remnants were present in WR causing greater refraction of light.

Annealing increased light transmittance in waxy starches, except WR, even though annealing decreased granule swelling in waxy starches (Table 4.4). It could be that higher temperature (boiling temperature) would have affected the structural changes that occurred on annealing in a way that facilitates more water penetration and granule swelling. The acetylation treatment increased the light transmittance in all waxy starches due to increased swelling (Table 4.4). This increase may be attributed to the introduction of hydrophilic substituting groups, which influence water penetration and absorption on the starch granules, leading to greater swelling of starch granules and transmittance of light which retained the water molecules to form hydrogen bonds

in starch granules, resulting in a higher light transmittance (Craig, Maningat, Seib, & Hoseney, 1989; Betancur, Chel, & Cañizares, 1997). Furthermore, the introduction of acetyl groups causes repulsions between adjacent starch molecules (Han et al., 2012; Wani et al., 2012) and thus reduces inter-chain association leading to higher light transmittance (Sun et al., 2016). Increase in acetyl content increased light transmittance in waxy starches. However, significant increases were observed only in WC and WP, while WR and WB showed only marginal increases. Dual modification treatment showed increased light transmittance compared to native and annealed counterparts as a result of increased granule swelling (Table 4.4). The light transmittance of dual modified WR was significantly higher ($p<0.05$) than acetylated WR, while in WP it was significantly higher ($p<0.05$) than acetylated WP with 10% acetic anhydride. In WC, the light transmittance of dual modifications was not significantly different ($p>0.05$) from that of acetylated WC with 10% acetic anhydride, while dual modified WB showed no significant difference ($p>0.05$) in light transmittance compared to acetylated WB. Variations in the extent of structural changes occurred in dual modification of waxy starches could be attributed to the differences in turbidity of starch pastes.

The transmittances of all the suspensions of native and modified starches decreased during storage. The light transmittance of native and annealed starches decreased greatly during the first 24 h of storage, and this decrease was more evident in WC and WP which had higher light transmittance values at the beginning (day 0). The rapid decrease in light transmittance during the first 24 h could be attributed to the network formation resulting from the interaction between starch chains that were leached out of the granules during gelatinization. This initial network formation was attributed to the amylose-amylose interactions (Ambigaipalan et al., 2013), however, waxy starches are mainly composed of amylopectin, and hence starch chain

interactions could be only possible between amylopectin chains. In acetylated and dual modified WC and WP starches, light transmittance decreased after 24 h of storage, except for acetylated WC with 10% acetic anhydride, where it remained unchanged. However, in WR and WB, a decrease in light transmittance after 24 h of storage was only observed in acetylated WB with 5 and 20% acetic anhydride and dual modified WR. The initial (day 0) light transmittance values of acetylated and dual modified WB (14.8-18.6%) and WR (9.1-11.0%) were significantly lower than that of WC (58.2-65.3%) and WP (80.0-87.3%), and hence the changes during the storage of acetylated and dual modified WB and WR were only marginal and was not detectable when measuring light transmittance. The light transmittance of acetylated and dual modified waxy starches was higher than that of native and annealed counterparts, except for acetylated WC and WB with 5% acetic anhydride which had lower values than those of their annealed counterparts.

After 24 h of storage, the decrease in light transmittance was only marginal throughout the storage time (7 days) in native and modified waxy starches, except for annealed WC and WP where the light transmittance decreased with storage time, indicating that amylopectin retrogradation is greater in annealed WC and WP. The light transmittance of acetylated corn and potato starches remained higher than their native counterparts and with higher acetyl (%) decrease in the light transmittance was slower during storage (20 days) (Singh et al., 2004). The light transmittance (%) increased with the DS, and it remained relatively high and decreased only slightly compared to the drastic decrease in light transmittance in native lotus rhizome starch during the storage time (Sun et al., 2016). A significant increase in paste clarity was also observed for potato and cassava starches after the acetylation process (Mbougueng et al., 2012). Higher light transmittance in acetylated and dual modified starches compared to native and annealed counterparts may have occurred due to lower levels of retrogradation which resulted

from the introduction of bulky acetyl groups that prevented the aggregation of amylopectin chains in the starch pastes (Singh et al., 2004; Sun et al., 2016).

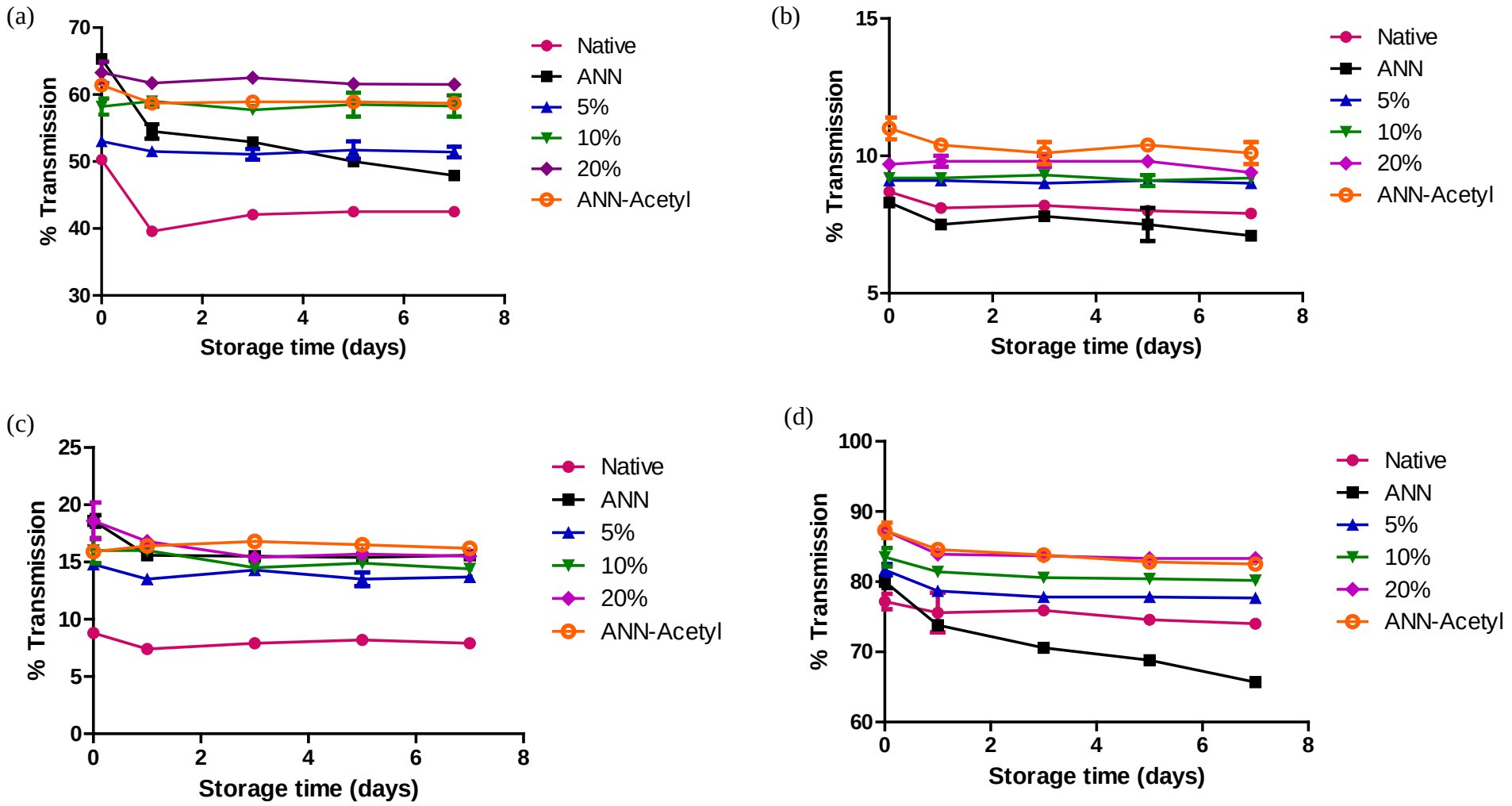



**Figure 4.10** Turbidity profiles of native, annealed (ANN) , acetylated (with 5,10, and 20% acetic anhydride), and annealed-acetylated waxy corn (a), waxy rice (b), waxy barley (c), and waxy potato (d) starches

#### 4.8.5.2 Differential scanning calorimetry (DSC)

The retrogradation transition temperatures and the melting enthalpies ($\Delta H_R$) of amylopectin re-crystallization are presented in Table 4.7. Retrogradation of starch depends on moisture content, and it has been reported that the maximum extent of starch re-crystallization occurs between 40 - 50% starch concentrations (Longton & LeGrys, 1981; Zeleznak & Hoseney, 1986). Therefore DSC measurements were carried out at 50% starch concentration. DSC measurements were taken after 24 h and 7 days of storage. Native and modified waxy cereal starches did not show endotherms after 24 h, indicating that retrogradation has not occurred during the first day of storage in waxy cereal starches. However, native and modified WP starches showed retrogradation endotherms at the first day of storage. Retrogradation enthalpies were observed in waxy corn (Liu & Thompson, 1998) and potato (Gunaratne & Hoover, 2002) starches after 24 h of storage.

Retrogradation endotherms appeared in all waxy starches after 7 days of storage expect for acetylated starches with 10 and 20% acetic anhydride and dual modified WR and WB. In WP, retrogradation transition temperatures and the melting enthalpies increased, and in waxy cereal starches, retrogradation endotherms appeared with storage time, indicating the progress of starch retrogradation over the storage period. Further, $T_c$-$T_o$ decreased after 7 days compared to 24 h of storage in WP, showing that variations in size, stability, and perfection of the crystallites formed during retrogradation have narrowed and crystallites have become more homogenous over the storage period.

In native starches $\Delta H_R$ were greater in WP compared to waxy cereal starches, since the amylopectin from B-type (potato) retrograde to a greater extent than amylopectin from A-type (cereal) starches (Silverio, Svensson, Eliasson, & Olofsson, 1996; Gunaratne & Hoover, 2002) as

a result of shorter average chain length distribution of A-type starches (Orford, Ring, Carroll, Miles, & Morris, 1987). Ward, Hoseney, and Seib (1994) and Würsch and Gumy (1994) postulated that an increase in the molar proportion of short chains (DP 6-9) inhibits retrogradation while an increased molar proportion of unit chains with DP 14–24 facilitates the extent of retrogradation. Even though there was no significant difference ($p>0.05$) in DP 13-24 chains (Table 4.1) between waxy starches in the current study, significant low ($p<0.05$) proportion of DP 6-12 would have contributed to the greater retrogradation in WP.

Retrogradation transition temperatures after 7 days of storage increased slightly in modified WC and WP compared to native counterparts, however differences between annealed, acetylated, and dual modified starches were only marginal suggesting that crystallites formed during storage have similar perfection. Further, only marginal changes were observed in retrogradation transition temperatures native, annealed and acetylated (with 5% acetic anhydride) WR and WB starches. However, significant changes were observed in $\Delta H_R$, where significantly lower enthalpies were observed in acetylated starches compared to native and annealed counterparts. Starch retrogradation involves re-aggregation of starch molecules in stored gels, where hydrogen bonding is used for re-crystallization, and acetylation restricts the hydrogen bonding as a result of inter and intra-molecular electrostatic repulsion in the starch molecules (Gonzalez & Pérez, 2002; Lawal & Adebowale, 2005). Increase in acetyl content caused a further decrease in $\Delta H_R$ in WC and WP, while endotherms were not observed for 10 and 20% acetic anhydride treatment for WR and WB, indicating that higher the acetyl groups greater the inhibition of retrogradation. Further, compared to WC and WP, retrogradation of acetylated WB and WR was limited and that can be attributed to the higher DS in acetylated WB and WR (Table 4.2), especially with 20% acetic anhydride treatment. In native and acetylated (DS 0.029-0.085) hulless barley

starches no significant endothermic peak of gels (stored for 7 days) were observed, indicating that retrogradation of starch gels was too little to be measured by DSC (Chang & Lv, 2017). Dual modification showed significantly lower ($p<0.05$) $\Delta H_R$ than native, annealed, and 5% acetic anhydride treatment in WC and WP, while endotherms were not observed in WR and WB. Compared to annealing, the dual modification is a promising technique to retard retrogradation in waxy starches.

**Table 4.7** Retrogradation transition temperatures and enthalpies of native, annealed, acetylated, and annealed-acetylated waxy starches[1*]

| Starch source | Retrogradation transition parameters | | | | |
|---|---|---|---|---|---|
| | $T_o$ (°C)[2] | $T_p$ (°C)[2] | $T_c$ (°C)[2] | $T_c$-$T_o$ (°C)[3] | ΔH (J/g)[3] |
| **After 24 h of storage**[4] | | | | | |
| **Waxy potato** | | | | | |
| Native | 51.50±0.04$^{cd}$ | 65.47±0.36$^{a}$ | 72.45±0.28$^{b}$ | 20.98±0.25$^{a}$ | 0.95±0.04$^{ab}$ |
| ANN | 52.09±0.11$^{bc}$ | 65.95±0.05$^{a}$ | 73.73±0.30$^{a}$ | 21.64±0.18$^{a}$ | 1.03±0.03$^{a}$ |
| 5% | 50.35±0.47$^{d}$ | 62.78±0.14$^{c}$ | 69.54±0.42$^{d}$ | 19.19±0.06$^{b}$ | 0.88±0.01$^{bc}$ |
| 10% | 51.49±0.66$^{cd}$ | 63.47±0.36$^{bc}$ | 69.86±0.16$^{d}$ | 18.37±0.50$^{b}$ | 0.75±0.04$^{cd}$ |
| 20% | 53.15±0.13$^{b}$ | 63.48±0.13$^{bc}$ | 70.52±0.08$^{d}$ | 17.37±0.04$^{c}$ | 0.63±0.02$^{d}$ |
| ANN-Acetyl | 54.62±0.08$^{a}$ | 64.37±0.23$^{b}$ | 71.58±0.14$^{c}$ | 16.96±0.06$^{c}$ | 0.67±0.04$^{d}$ |
| **After 7 days of storage** | | | | | |
| **Waxy corn** | | | | | |
| Native | 64.49±0.34$^{c}$ | 69.92±0.34$^{c}$ | 74.27±0.08$^{c}$ | 9.77±0.42$^{ab}$ | 3.35±0.20$^{a}$ |
| ANN | 66.04±0.25$^{ab}$ | 71.14±0.17$^{ab}$ | 76.41±0.22$^{a}$ | 10.37±0.47$^{a}$ | 3.37±0.08$^{b}$ |
| 5% | 66.28±0.37$^{ab}$ | 71.51±0.42$^{ab}$ | 75.37±0.23$^{b}$ | 9.09±0.10$^{ab}$ | 2.16±0.21$^{b}$ |
| 10% | 66.60±0.36$^{a}$ | 71.77±0.14$^{a}$ | 76.28±0.21$^{a}$ | 9.68±0.60$^{ab}$ | 1.30±0.12$^{c}$ |
| 20% | 65.87±0.03$^{ab}$ | 70.45±0.13$^{bc}$ | 74.48±0.01$^{c}$ | 8.61±0.00$^{b}$ | 1.02±0.01$^{c}$ |
| ANN-Acetyl | 65.31±0.11$^{bc}$ | 69.78±0.31$^{c}$ | 74.00±0.16$^{c}$ | 8.70±0.00$^{b}$ | 1.47±0.07$^{c}$ |
| **Waxy rice** | | | | | |
| Native | 64.37±0.23$^{ab}$ | 72.02±0.05$^{a}$ | 78.01±0.14$^{a}$ | 13.80±0.08$^{a}$ | 0.43±0.04$^{a}$ |
| ANN | 63.62±0.38$^{b}$ | 70.26±0.08$^{b}$ | 78.10±0.17$^{a}$ | 14.48±0.55$^{a}$ | 0.26±0.03$^{b}$ |
| 5% | 65.26±0.06$^{a}$ | 71.00±0.30$^{b}$ | 76.87±0.21$^{b}$ | 11.61±0.27$^{b}$ | 0.18±0.04$^{b}$ |
| **Waxy barley** | | | | | |
| Native | 66.81±0.16$^{a}$ | 71.23±0.04$^{b}$ | 74.52±0.29$^{a}$ | 7.60±0.45$^{c}$ | 0.99±0.01$^{a}$ |
| ANN | 65.59±0.23$^{a}$ | 70.25±0.21$^{a}$ | 74.43±0.17$^{a}$ | 8.85±0.06$^{a}$ | 1.72±0.04$^{a}$ |
| 5% | 66.31±0.14$^{ab}$ | 70.39±0.25$^{a}$ | 74.16±0.21$^{a}$ | 7.85±0.35$^{b}$ | 0.58±0.08$^{b}$ |

**Table 4.7** *(continued)*

| Starch source | Retrogradation transition parameters | | | | |
|---|---|---|---|---|---|
| | $T_o$ ($^oC$)[2] | $T_p$ ($^oC$)[2] | $T_c$ ($^oC$)[2] | $T_c$-$T_o$ ($^oC$)[3] | $\Delta H_R$ (J/g)[3] |
| **Waxy potato** | | | | | |
| Native | 66.98±0.08[b] | 73.19±0.25[b] | 79.52±0.07[a] | 12.60±0.01[ab] | 5.50±0.25[a] |
| ANN | 67.39±0.25[ab] | 74.01±0.08[a] | 80.41±0.28[a] | 13.02±0.03[a] | 5.04±0.04[a] |
| 5% | 67.94±0.06[ab] | 74.14±0.10[a] | 80.07±0.21[a] | 12.13±0.15[bc] | 3.92±0.09[b] |
| 10% | 68.37±0.21[a] | 74.30±0.07[a] | 80.53±0.48[a] | 12.16±0.27[bc] | 3.30±0.13[c] |
| 20% | 68.46±0.40[a] | 74.38±0.15[a] | 80.38±0.43[a] | 11.92±0.04[c] | 3.12±0.04[c] |
| ANN-Acetyl | 68.45±0.42[a] | 74.31±0.30[a] | 80.11±0.61[a] | 11.66±0.18[c] | 3.26±0.03[c] |

[1]All data represent the mean of triplicates. Values followed by the same superscript in each column in each starch source are not significantly different ($p>0.05$).

[2]$T_o$,$T_p$, and $T_c$ indicate the onset, peak, and end temperature, respectively.

[3]($T_c$-$T_o$) and $\Delta H_R$ indicates the temperature range and enthalpy of retrogradation, respectively.

[4]Retrogradation endotherms after 24h storage were only observed in WP.

[*]5, 10, and 20% represent acetylated starch with 5, 10, and 20% acetic anhydride, respectively.

## 4.9 Mass spectrometric analysis

### 4.9.1 Analysis of structure of native and modified starches after enzyme digestion

The enzymes (α-amylase and amyloglucosidase) can cleave the glycosidic linkages in starch molecules giving rise to lower molecular weight fragments. Mass spectrometry (MS) can be used to identify the distribution of acetyl groups in the hydrolyzed starch molecules. Acetyl groups can be partitioned between amorphous and crystalline regions of the amylopectin structure, and differences in the reactivities of the –OH groups at C6, C2, and C6 would lead to differences in the substitution distribution of acetyl groups, Furthermore, these differences in substitution distribution can be vary depending on the starch type and the DS in acetylated and dual modified starches. MS can be used to identify the differences in the substitution distribution in different starches and these finding will be helpful to relate the variation in their physicochemical properties.

ESI-MS spectra of native and annealed waxy starches showed a series of protonated and sodiated ($Na^+$ containing) molecules at *m/z* 182.4, 203.1, 342.5, 365.4, 504.3, 663.2, and 823.8 (Fig 4.11 and Table 4.8). Even though different peaks appeared at 1000-2000 *m/z* range, peaks which represent starch hydrolysates were not identified.

The ESI-MS spectra of acetylated and dual modified waxy starches showed a series of sodiated molecules (which contained acetyl groups) other than the peaks identified in native starches. Acetylation with 5% acetic anhydride resulted in sodiated molecules at *m/z* 407.3, 568.7, 611.1, 772.9, 893.0, 1055.8, 1098.0, 1138.2, 1259.1, 1301.6 and these peaks represented acetylation with 1-3 acetyl groups. The peak at around *m/z* 1259 appeared only in WC and WB, while the peak at around *m/z* 1302 appeared only in WB and WR. Acetylation with 10 and 20% acetic

anhydride and dual modification resulted in few new sodiated molecules at *m/z* 730.6, 815.3, 892.5, 934.1, 977.4, and 1463.3 (Fig 4.12) with various numbers of acetyl groups attached (Table 4.9). However, the appearance of the peaks varied with acetyl content and starch source, thus indicating the difference in the distribution of acetyl groups. Since the enzyme hydrolysates of acetylated and dual modified starches contain starch chains with DP>5, compared to native starch hydrolysates where the identified hydrolysates had a maximum of DP 5, it was evident that acetyl groups hinder the enzyme digestion and hence retaining starch chains with higher DP.

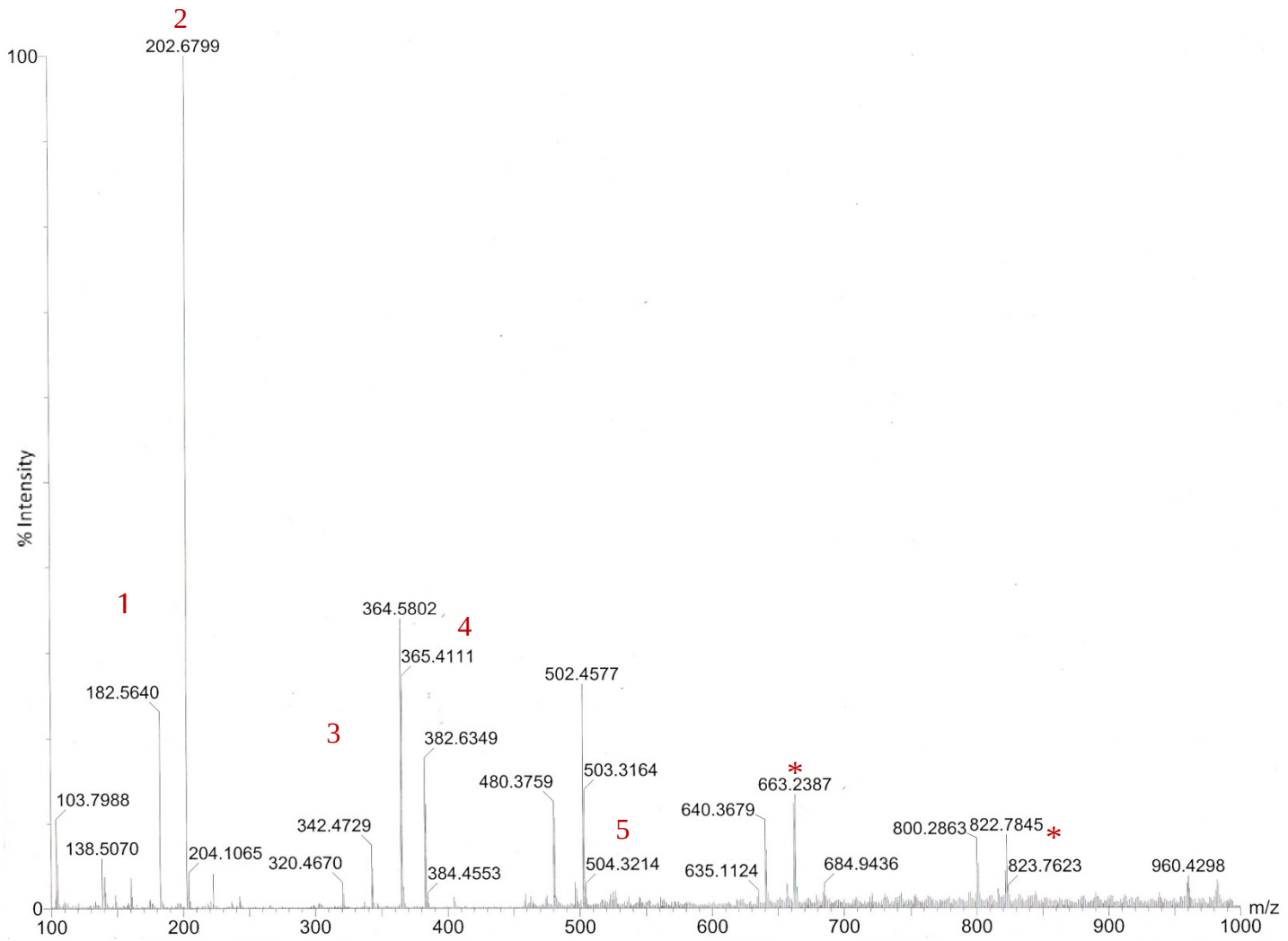
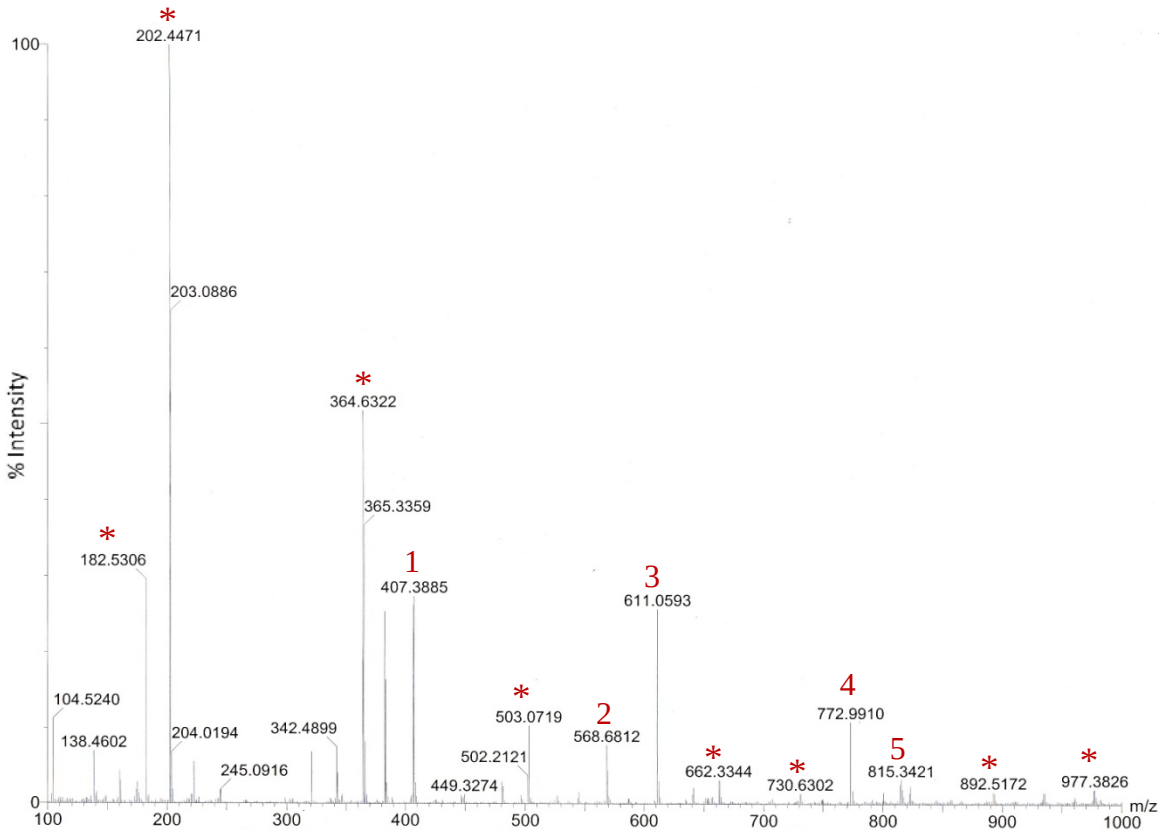



**Figure 4.11** Triplequadrupole ESI-MS (positive ion mode) spectrum of hydrolyzed native WR starch recorded from *m/z* 100-1000

**Table 4.8** Identification of starch hydrolysates from native and annealed waxy starches using Triplequadrupole ESI-MS/MS

| Precursor ion | Assigned formula | Molecular weight (Da) | Observed ion (*m/z*) | | CID-MS/MS product ions |
|---|---|---|---|---|---|
| 1 | $[C_6H_{12}O_6+H]^+$ | 181.07 | 182.4 | $[M+H]^+$ | - |
| 2 | $[C_6H_{12}O_6+Na]^+$ | 203.05 | 203.1 | $[M+Na]^+$ | - |
| 3 | $[C_{12}H_{22}O_{11}+H]^+$ | 343.12 | 342.5 | $[M_2+H]^+$ | 138.4, 182.2, 203.1, 297.7 |
| 4 | $[C_{12}H_{22}O_{11}+Na]^+$ | 365.10 | 365.4 | $[M_2+Na]^+$ | 202.9, 244.9, 274.8, 304.6 |
| 5 | $[C_{18}H_{32}O_{16}+H]^+$ | 505.17 | 504.3 | $[M_3+H]^+$ | 138.6, 182.1, 298.0, 342.6 |

(a)




(b)

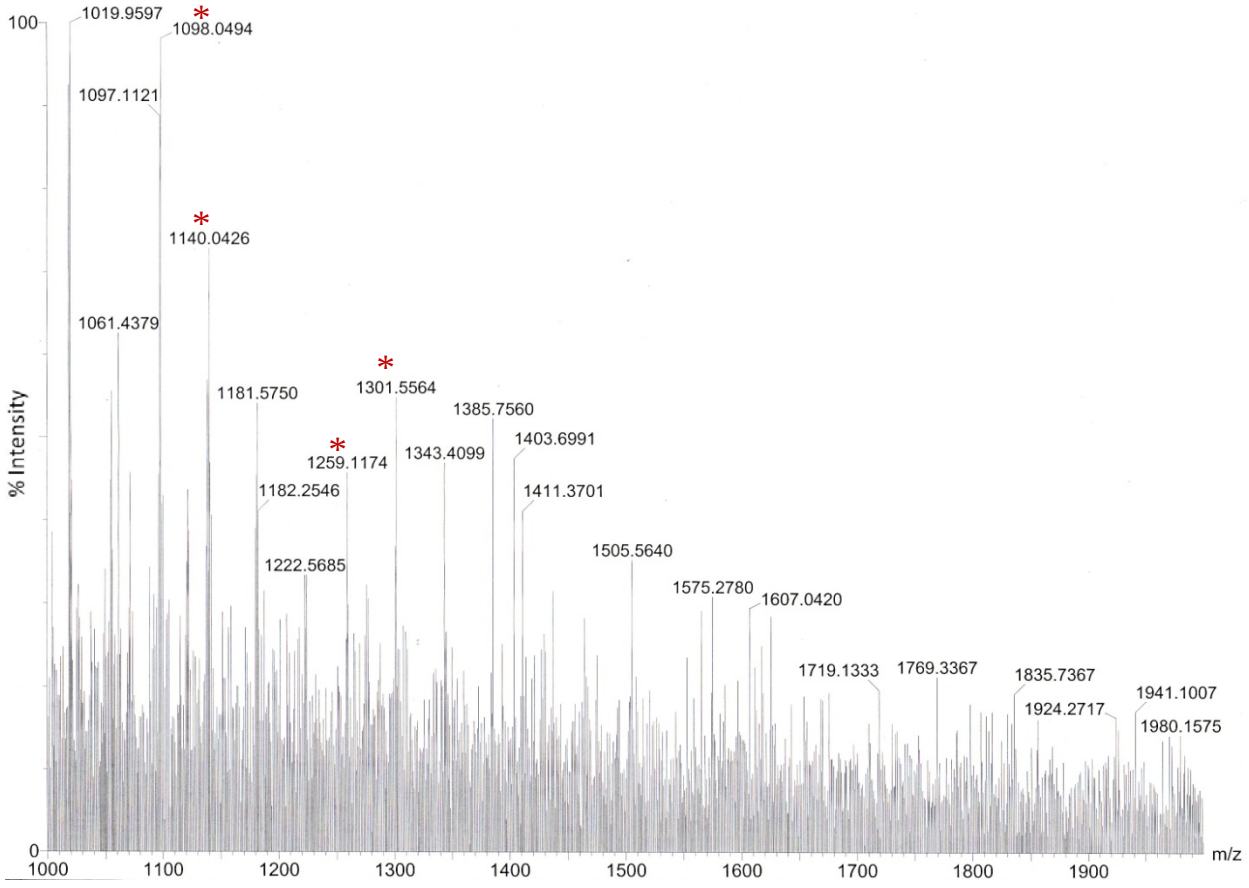



**Figure 4.12** Triplequadrupole ESI-MS (positive ion mode) spectra of hydrolyzed acetylated (20% acetic anhydride) WR starch recorded from *m/z* (a) 100-1000 (b) 1000-2000

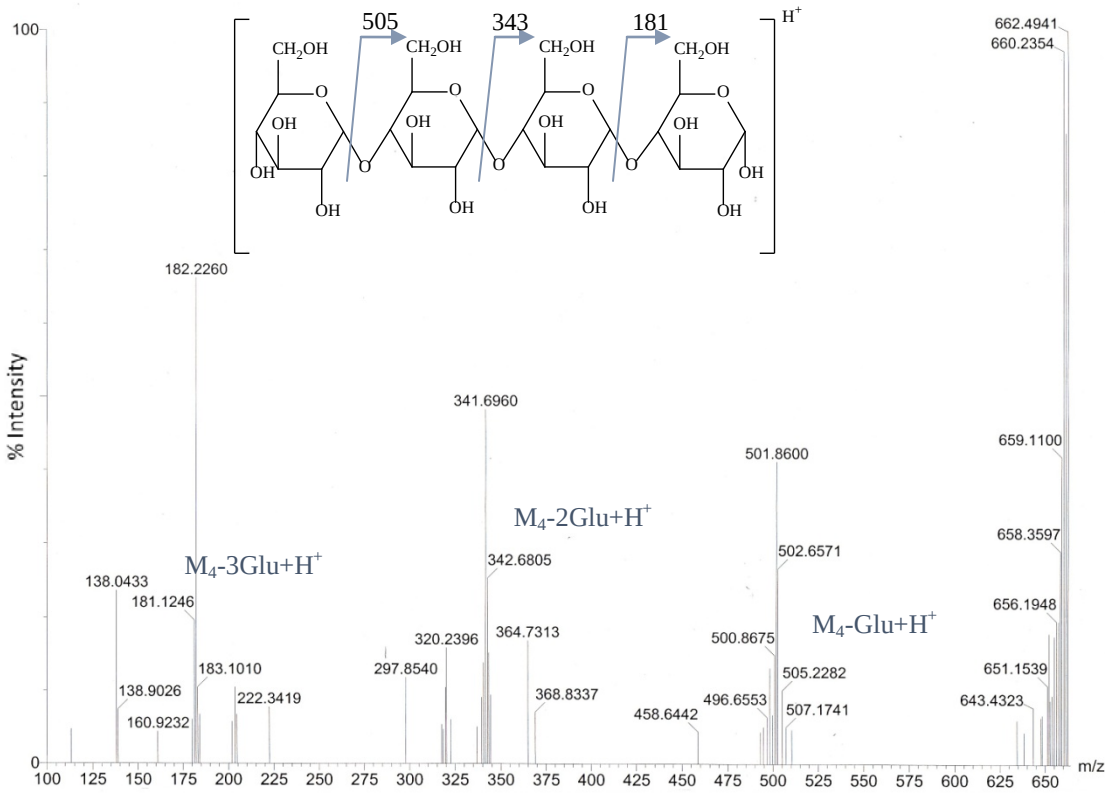
**Table 4.9** Identification of starch hydrolysates from acetylated and annealed-acetylated waxy starches using Triplequadrupole ESI-MS/MS

| Precursor ion | Assigned formula | Molecular weight (Da) | Observed ion (*m/z)* | | CID-MS/MS product ions |
|---|---|---|---|---|---|
| 1 | $[C_{14}H_{24}O_{12}+Na]^+$ | 407.11 | 407.3 | $[M_2Ac-H+Na]^+$ | 202.4, 243.6, 286.5, 239.8, 345.8, 389.6 |
| 2 | $[C_{20}H_{34}O_{17}+Na]^+$ | 569.16 | 568.7 | $[M_3Ac-H+Na]^+$ | 202.2, 363.7, 388.5, 508.2, |
| 3 | $[C_{22}H_{36}O_{18}+Na]^+$ | 611.17 | 611.1 | $[M_3Ac_2-2H+Na]^+$ | 201.6, 344.8, 406.8, 448.9, 509.5, 549.7 |
| 4 | $[C_{28}H_{46}O_{23}+Na]^+$ | 773.23 | 772.9 | $[M_4Ac_2-2H+Na]^+$ | 364.0, 387.9, 405.4, |
| 5 | $[C_{30}H_{48}O_{24}+Na]^+$ | 815.24 | 815.3 | $[M_4Ac_3-3H+Na]^+$ | 202.7, 406.2, 448.4, 609.6, 653.0 |

The CID-MS/MS spectra obtained for native and annealed starch hydrolysates showed glycosidic and cross-ring cleavage fragments. Glycosidic fragments were observed in all waxy starches, while cross-ring fragments varied. Fragments of $[M+H]^+$ ions were mainly glycosidic cleavage (Fig 4.13a). Product ions obtained from the CID-MS/MS analysis of the $[M+Na]^+$ ions were mainly formed by glycosidic cleavage and by losses of 60 and 120 Da (Fig 4.13b), which were attributed to neutral molecules of $C_2H_4O_2$ and $C_4H_8O_4$ (Hofmeister, Zhou, & Leary, 1991; Asam & Glish, 1997; Čmelík & Chmelík, 2010). The predominant mechanism of CID-MS/MS fragmentation of cationized oligosaccharides involves a series of retro-aldol reactions occurring on the reducing end of the molecule (Zhou, Ogden, & Leary, 1990; Hofmeister et al., 1991).

The CID-MS/MS spectra of precursor sodiated molecules which contained acetyl groups resulted from glycosidic cleavage and from few cross-rings fragmentations. The CID-MS/MS spectra of precursor ion $[M_2Ac-H+Na]^+$ resulted loss of 18, 60, and 120 Da other than glycosidic cleavage. However, it was difficult to determine the location of the acetyl group with the fragmentation obtained. The CID-MS/MS spectra of precursor ion $[M_3Ac_2-2H+Na]^+$ resulted fragment ions of $[M_2Ac_2-2H+Na]^+$ and $[M_2Ac-H+Na]^+$ indicating that two acetyl groups are not located in the same glucose molecule, rather they are located in two different glucose molecules. This was confirmed with CID-MS/MS spectra obtained for $[M_4Ac_3-3H+Na]^+$, where fragment ions contained loss of glucose and loss of glucose together with an acetyl group (Fig 4.14). However, the CID-MS/MS spectra did not provide details regarding the exact location ($C_6$, $C_2$ or $C_3$) of acetyl groups.

(a)




(b)

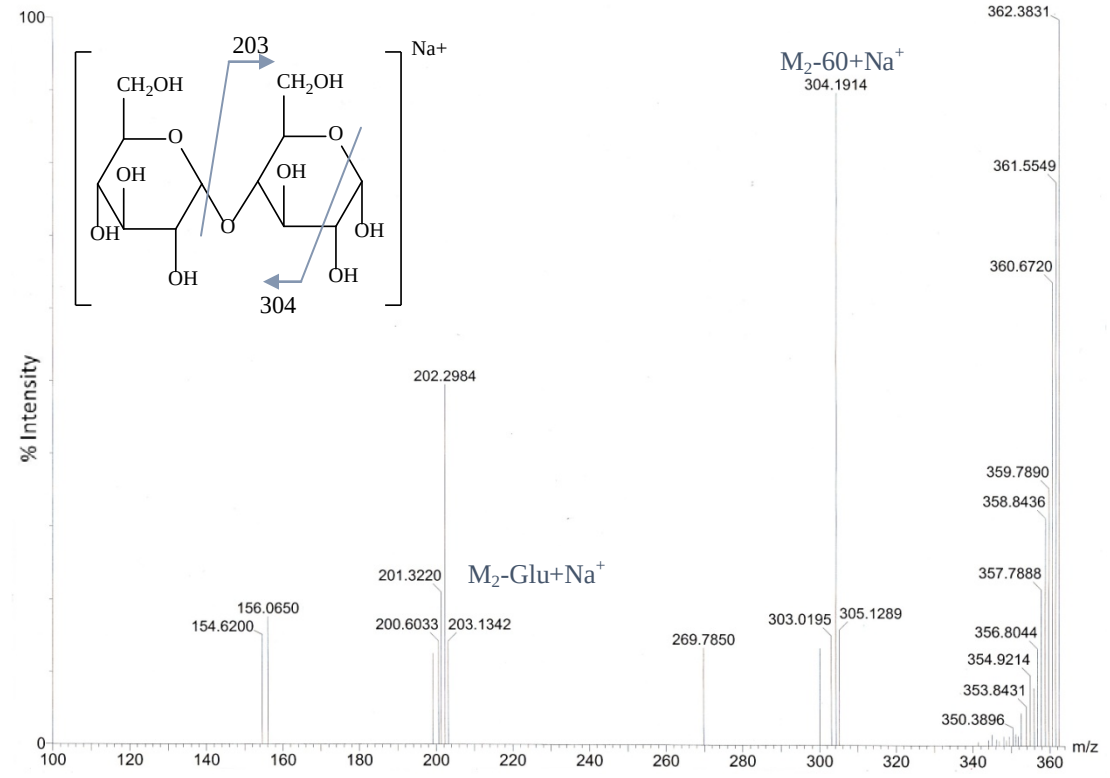



**Figure 4.13** Triplequadrupole ESI-MS/MS of the precursor ion $[M_4+H]^+$ at *m/z* 663.3 (a) and $[M_2+Na]^+$ at *m/z* 365.4 (b) isolated from hydrolyzed native WR starch

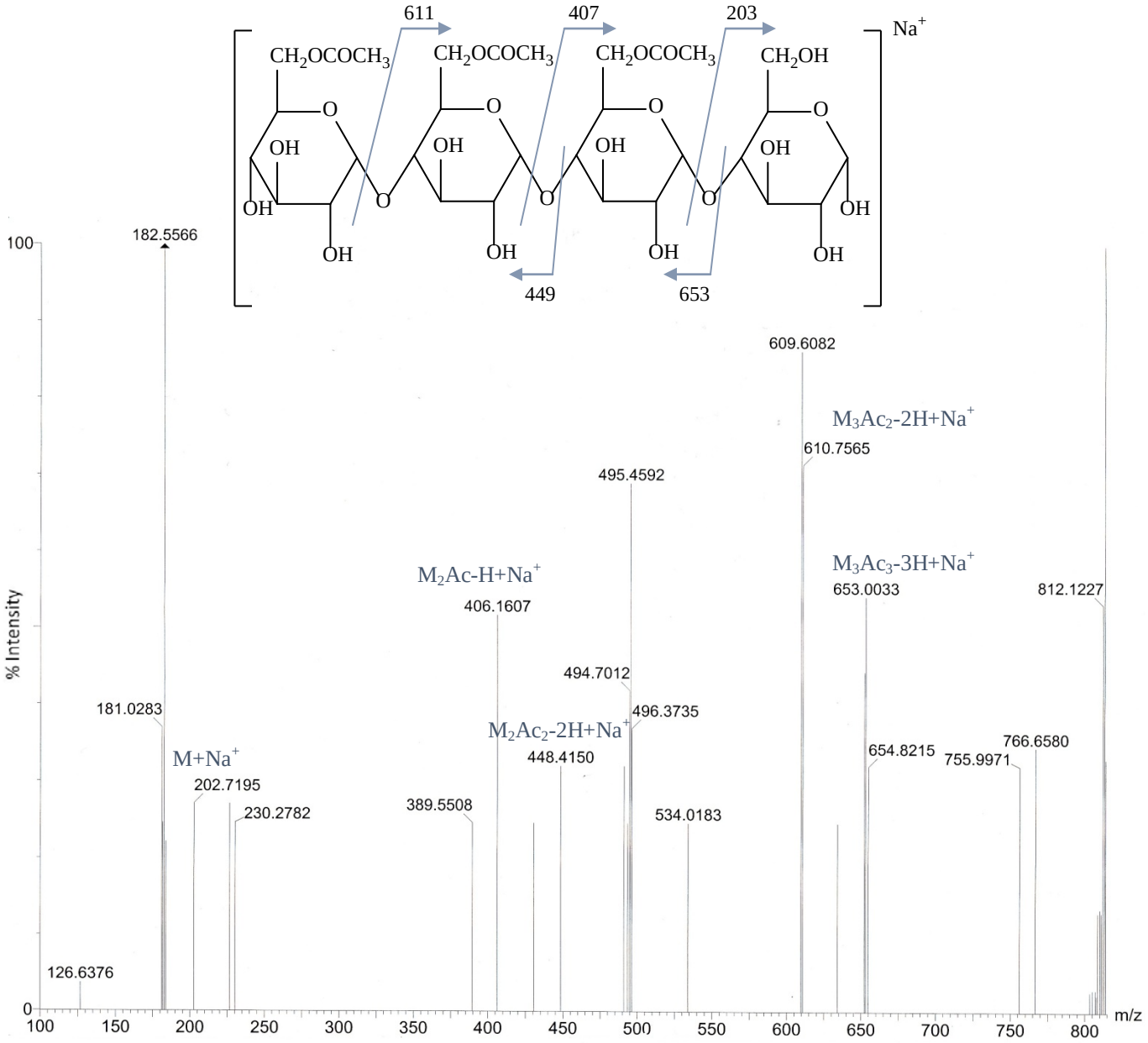


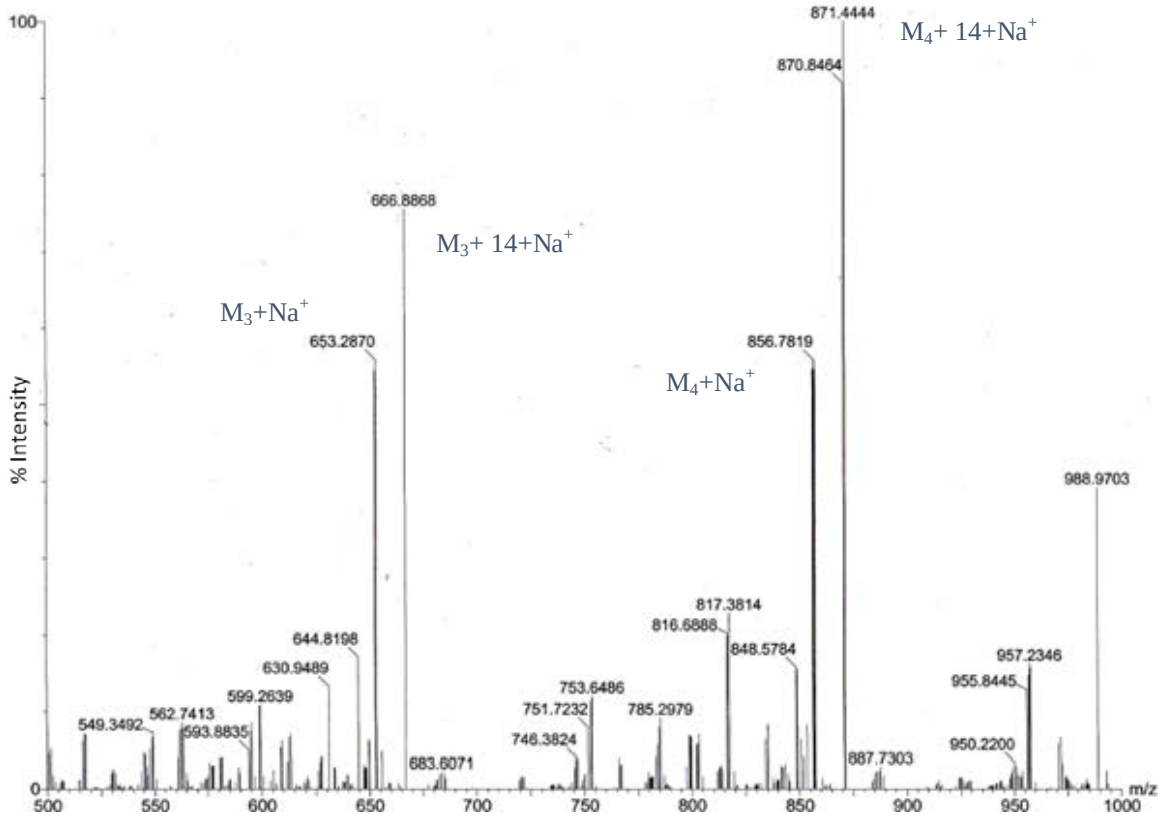

**Figure 4.14** Triplequadrupole ESI-MS/MS of the precursor ion $[M_4Ac_3\text{-}3H+Na]^+$ at *m/z* 815.3 isolated from hydrolyzed acetylated (20% acetic anhydride) WR starch

### 4.9.2 Analysis of structure of permethylated native starches

Native starch does not dissolve in water or organic solvent, and hence it cannot be used in ESI-MS analysis. When native starch is derivatized by methylation, it is dissolved in solvents and then can be used in ESI-MS analysis. Pure amylose and amylopectin were also permethylated in order to investigate the structure of starch using ESI-MS. Mass spectra of permethylated amylose, amylopectin, as well as waxy starches showed a series of sodiated molecules in a *m/z* range of 204-2300, which represented maximum DP of 11. However pure amylose and amylopectin had maximum identified *m/z* at 3919 (DP 19) and 2695 (DP 13). The response decreased with increasing mass/degree of polymerization. The sodiated molecule series had peaks appearing at intervals of 204 Da, indicating that glucose molecules have fully methylated. Each point of the series had two distinct peaks with a difference of 14 Da; $[M+Na]^+$ and $[M+14+Na]^+$, which is an extra methyl group (Fig 4.15). The peaks with various DP can be due to the presence of starch chains with various lengths and/or as a result of in-source decay during the ESI-MS analysis.

The CID-MS/MS spectra of both $[M+Na]^+$ and $[M+14+Na]^+$ precursor ions gave glycosidic cleavage and loss of 32, 48, and 134 Da which can be attributed to $CH_3OH$, HCHO and $H_2O$, and $C_3O_4H_{10}$ (Fig 4.16). Compared to CID-MS/MS spectra of acetylated and native starch hydrolysates more fragmentation was observed for permethylated starches, indicating that permethylation increases the sensitivity of starch for ESI-MS analysis. However, information obtained from analyzing CID-MS/MS spectra of permethylated amylose, amylopectin, and waxy starches could not be used to differentiate between α-D-(1→4) and α-D-(1→6) linkages.

(a)




(b)

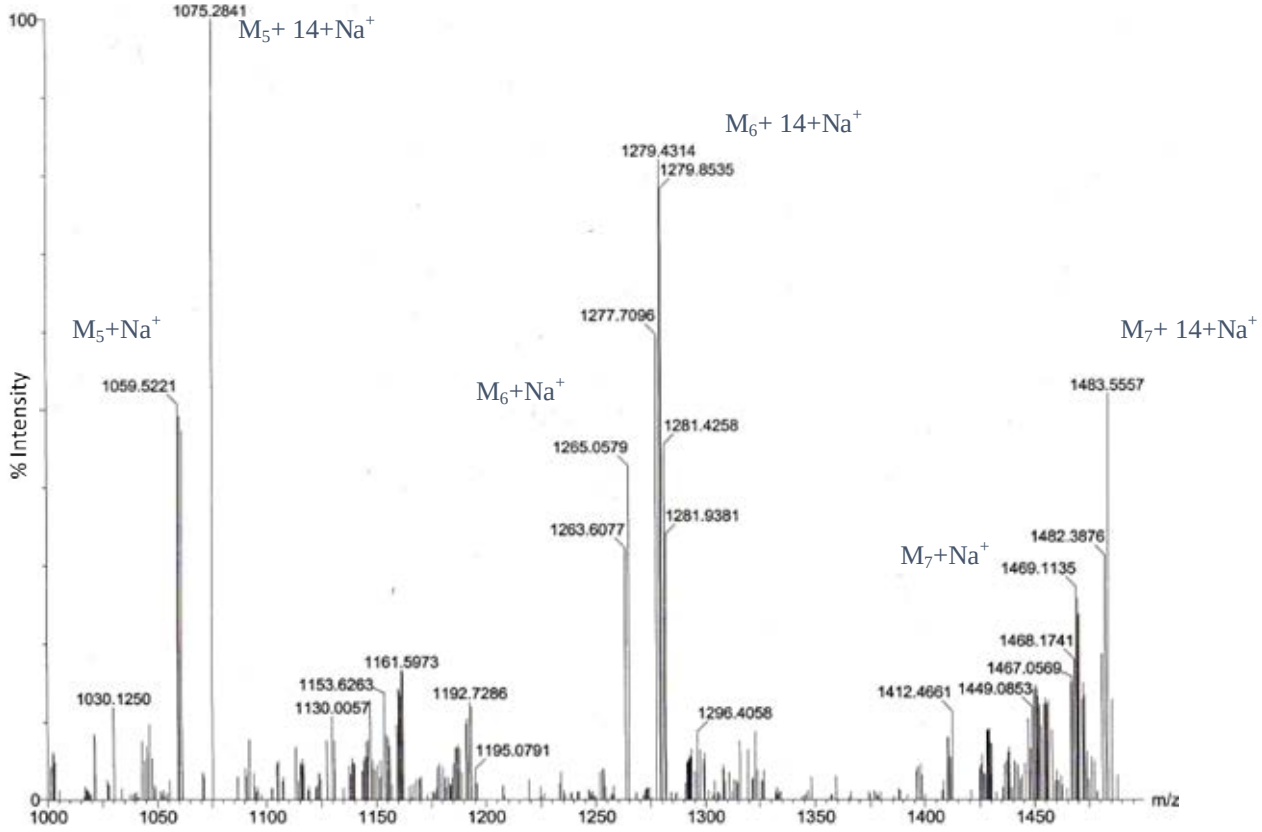



**Figure 4.15** Triplequadrupole ESI-MS (positive ion mode) spectra of permethylated WR starch recorded from *m/z* 500-1000 (a) 1000-1500 (b)

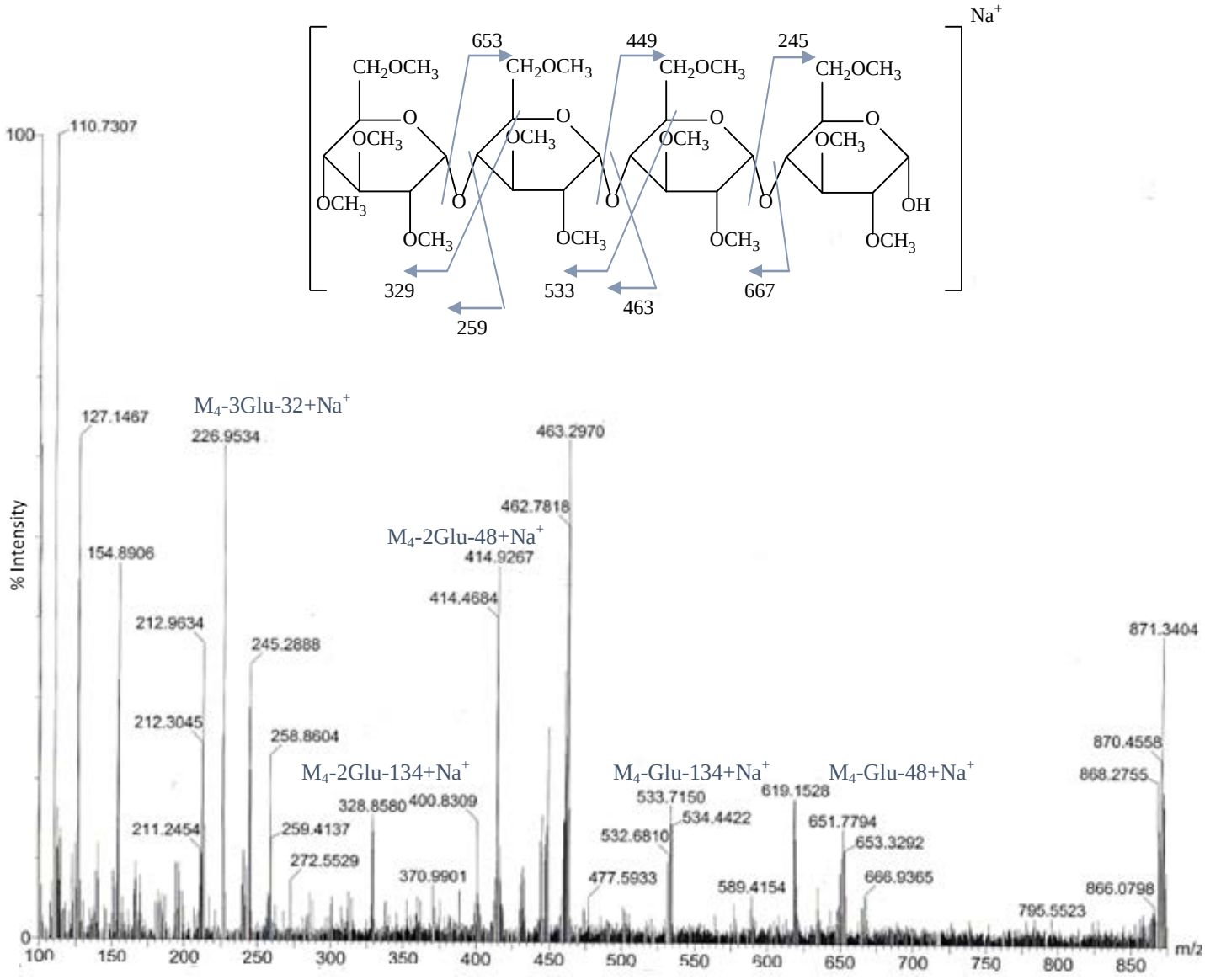



**Figure 4.16** Triplequadrupole ESI-MS/MS of the precursor ion $[M_4+Na]^+$ at *m/z* 871.3 isolated from permethylated WR starch

# CHAPTER 5

# SUMMARY AND CONCLUSIONS

## 5.1 Summary

The structural analysis of native waxy starches from different botanical origins showed that variations occur with respect to phosphorous content, amylopectin chain length distribution, and packing of crystallites and these structural variations lead to changes in their properties. These differences had a major impact on granule swelling (WP>WB>WR>WC), gelatinization transition temperatures (WC>WP>WR>WB), enthalpy of gelatinization (WP>WC>WB>WR), rapidly digestible starch (WR>WC~WB>WP), slowly digestible starch (WC>WB>WR>WP), resistant starch (WP>WB>WR~WC), and the extent of light transmittance and retrogradation enthalpy (WP>WC>WB~WR).

Annealing did not affect the morphological characteristics, crystalline pattern, and gelatinization enthalpy of waxy starches. However, annealing increased gelatinization transition temperatures and decreased gelatinization transition temperature range and granule swelling, and a greater impact was observed in WP. Even though the tested starches were devoid of amylose, the extent of structural changes occuring during annealing varied depending on the structure of native waxy starches. It was observed that crystallites in native WP were less perfect than in other starches since the phosphate groups interfered with the formation of ordered structures, hence showing the pronounced effects of annealing. Annealing increases the mobility of amylopectin chains which causes reorientation of phosphate groups, allowing more amylopectin groups to interact, thus forming more ordered structures. Furthermore, only slight changes were observed in acid hydrolysis and enzyme susceptibility of waxy cereal starches, whereas the effects were distinct in WP upon annealing, indicating that a greater extent of crystalline perfection occurred in WP. Annealing caused an increase in light transmittance in WC and WB while it decreased in WP.

However, retrogradation characteristics measured by DSC showed only marginal changes on annealing. Overall, annealing effect on structure and functionality of starches which are devoid of amylose was marginal except for WP starch which had less perfect crystallites in the native structure. This indicated that only the crystalline perfection occurring in the fine structure in the absence of amylose was not sufficient to make significant functionality changes.

Acetylation with various amounts of acetic anhydride (5, 10, and 20%) resulted in starch acetates with varying DS. Granule morphology slightly changed upon acetylation showing rough granule surface, slight fragmentation, and indentation. $^{1}$H NMR and FTIR analysis showed appearance of peaks and bands which were attributed to the introduction of acetyl groups. Even though the X-ray diffraction pattern did not change, relative crystallinity decreased with acetylation indicating the disruption of packing of crystallites upon acetylation. These structural changes were more intense when DS increased in all waxy starches. Acetylation increased granule swelling throughout the temperature range and decreased gelatinization transition temperatures and enthalpy compared to native counterparts, and these changes were more evident with increasing DS. Acetyl groups inhibited the inter-chain association of starch chains facilitating granule hydration and these extensively hydrated granules required less energy to reach gelatinization. Acetylation decreased RDS and SDS levels and increased RS contents in waxy starches, and highest RS contents were obtained with 20% acetic anhydride treatment since the introduction of acetyl groups could change the spatial structure of starch molecules altering the binding sites between α-amylase and starch molecule resulting in greater resistance. Acetylation decreased the extent of retrogradation. The light transmittance of acetylated waxy starches remained higher and significantly lower retrogradation enthalpies observed after 7 days of storage in acetylated starches compared to their native counterparts. Furthermore, retrogradation endotherms did not

appear in acetylated WR and WB starches with 10 and 20% acetic anhydride, indicating the low extent of retrogradation.

Annealing followed by acetylation with 10% acetic anhydride resulted in a significant increase in acetyl percentage (Ac%) and DS in all waxy starches indicating that structural changes occurred during annealing facilitates the introduction of acetyl groups to the starch chains. Dual modification showed morphological and structural changes that were similar to acetylated starches, but to different extents. However, relative crystallinity was lower than that of acetylated starches with 20% acetic anhydride. Dual modification increased granule swelling but to a restricted extent compared to acetylated starches. The swelling factor (SF) values of dual modified waxy cereal starches were not significantly different ($p>0.05$) to that of acetylated starches with 5% acetic anhydride throughout the temperature range except at 60$^{o}$C in WC. In WP, at 60 and 70$^{o}$C, SF values of dual modification were not significantly different ($p>0.05$) from that of annealed WP, indicating that crystalline perfection on annealing would have overcome the weakening of bonds by acetylation at 60 and 70$^{o}$C. Gelatinization transition temperatures of dual modified waxy starches were higher than that of native and acetylated starches and were not significantly different ($p>0.05$) from the gelatinization temperatures of annealed starches except for $T_o$ in WR and WB. However, $\Delta H$ of annealed acetylated starches were significantly lower ($p<0.05$) than native, annealed, and acetylated starches indicating that crystalline perfection caused by annealing made the crystallites more stable, but it was not sufficient to prevent disruption of packing of crystals during the dual modification. The dual modification resulted in a significant decrease ($p<0.05$) in RDS and SDS contents and a significant increase ($p<0.05$) in RS levels compared to native and annealed counterparts. Except for WR, acetylation of annealed starches had increased the RS content which was similar to or

higher than that of 20% acetic anhydride treatment. Dual modification treatment showed increased light transmittance compared to native, annealed, and acetylated starch with 5 and 10% acetic anhydride as a result of increased granule swelling. Furthermore, $\Delta H_R$ of dual modification was significantly lower ($p<0.05$) than native, annealed, and 5% acetic anhydride treatment in WC and WP, and retrogradation endotherms were not observed in dual modified WR and WB.

Triplequadrupole ESI-MS analysis of native and modified hydrolyzed waxy starches showed that the acetylation and dual modification resulted in starch hydrolysates with higher DP (DP>5) compared to native starch hydrolysates as a result of hindering the enzyme attack by acetyl groups. In acetylated and dual modified starches several peaks of starch hydrolysates were identified containing 1-3 acetyl groups, and tandem mass spectrometry analysis showed that when there were two acetyl groups, those were not located in the same glucose molecule, rather they were located in two different glucose molecules. ESI-MS analysis of permethylated amylose, amylopectin, as well as waxy starches showed a series of sodiated molecules in a *m/z* range of 204-2300, which represented maximum DP of 11. Furthermore, ESI-MS/MS analysis of precursor sodiated molecules resulted in more product ions than acetylated and native starch hydrolysates, indicating that permethylation increases the sensitivity of starch for ESI-MS analysis.

Annealing followed by acetylation treatment is a novel starch modification technique which was demonstrated to improve physicochemical properties with less chemical usage compared to single acetylation treatment. Since dual modification restricts granule swelling and gelatinization, it is evident that dual modified starches have gained thermal stability and hence can potentially be used in foods which are processed at relatively high temperatures in order to improve their textural characteristics. Dual modification treatment significantly decreased

retrogradation in waxy starches, indicating their potential utilization in refrigerated and frozen foods such as frozen desserts, fruit pie fillings, gravies, and salad dressings, among other, where syneresis can be reduced to a greater extent and to maintain textural properties. Furthermore, the greater RS content after dual modification demonstrates its nutritional value as a low glycemic index food and hence its potential use as a dietary fiber. RS can be incorporatd to foods without altering their appearance and texture, because of its bland taste, white color, and microparticulate structure. Therefore, this study provides new insights to combine physical and chemical modification techniques to produce starch with desired functionality with a minimum use of chemicals.

## 5.2 Directions for future research

1. This study demonstrated that the structural organization of starch chains within the amorphous and crystalline domains was altered upon annealing, acetylation and dual modification, leading to changes in physicochemical properties. However, the dual modification can be optimized using various annealing conditions and acetic anhydride levels to achieve enhanced functionality.

2. The type and extent of changes occurring upon starch modification are influenced by starch source and starch composition. This study was conducted to evaluate the impact of modification of starches which were devoid of amylose and hence provided only a partial glimpse into the molecular mechanism(s) of annealing, acetylation, and especially the dual modification. In order to further understand the molecular mechanism(s) of these modifications,experiments must be conducted on starches that vary widely in their amylose content.

3. Modification of waxy starches with annealing/acetylation followed by other physical/chemical modification techniques such as heat moisture treatment, ultra-high pressure, microwave heating, cross-linking, and substitution may provide new insights for production of starches with enhanced properties and hence leading to novel food and industrial applications.

**References**


Adebowale, K. O., Afolabi, T. A., & Olu-Owolabi, B. I. (2005). Hydrothermal treatments of Finger millet (Eleusine coracana) starch. *Food Hydrocolloids*, 19(6), 974-983.

Alcázar-Alay, S. C., & Meireles, M. A. A. (2015). Physicochemical properties, modifications and applications of starches from different botanical sources. *Food Science and Technology*, 35(2), 215-236.

Alvani, K., Qi, X., & Tester, R. F. (2012). Gelatinisation properties of native and annealed potato starches. *Starch-Stärke*, 64(4), 297-303.

Ambigaipalan, P., Hoover, R., Donner, E., & Liu, Q. (2013). Retrogradation characteristics of pulse starches. *Food Research International*, 54(1), 203-212.

Ambigaipalan, P., Hoover, R., Donner, E., Liu, Q., Jaiswal, S., Chibbar, R., Nantanga, K.K.M., & Seetharaman, K. (2011). Structure of faba bean, black bean and pinto bean starches at different levels of granule organization and their physicochemical properties. *Food Research International*, 44(9), 2962-2974.

American Association of Cereal Chemists (2000). *Approved methods of the AACC (10th ed.)*. St. Paul, MN, USA.

Ao, Z., & Jane, J. L. (2007). Characterization and modeling of the A-and B-granule starches of wheat, triticale, and barley. *Carbohydrate Polymers*, 67(1), 46-55.

Appelqvist, I. A., & Debet, M. R. (1997). Starch-biopolymer interactions—a review. *Food Reviews International*, 13(2), 163-224.

Asam, M. R., & Glish, G. L. (1997). Tandem mass spectrometry of alkali cationized polysaccharides in a quadrupole ion trap. *Journal of the American Society for Mass Spectrometry*, 8(9), 987-995.

Ashogbon, A.O. & Akintayo, E.T. (2014). Recent trend in the physical and chemical modification of starches from different botanical sources: A review. *Starch/Stärke*, 66, 41–57

Atkin, N. J., Cheng, S. L., Abeysekera, R. M., & Robards, A. W. (1999). Localisation of amylose and amylopectin in starch granules using enzyme-gold labelling. *Starch-Stärke*, 51(5), 163-172.

Atwell, W. A., Hood, L., Lineback, D., Varriano-Marston, E., & Zohel, H. (1988). The terminology and methodology associated with basic starch phenomenon. *Cereal Foods World*, 33, 306–311.

Baldwin, P. M. (2001). Starch granule-associated proteins and polypeptides: A review. *Starch-Stärke*, 53(10), 475-503.

Banks, W., & Greenwood, C. T. (1971). Amylose: a non-helical biopolymer in aqueous solution. *Polymer*, 12(2), 141-145.

Bao, J., Corke, H., & Sun, M. (2004). Genetic diversity in the physicochemical properties of waxy rice (Oryza sativa L) starch. *Journal of the Science of Food and Agriculture*, 84(11), 1299-1306.

BeMiller, J.N., & Huber, K.C. (2015). Physical modification of food starch functionalities. *Annual Review of Food Science & Technology*, 6, 19-69.

BeMiller, J. N. (2011). Pasting, paste, and gel properties of starch–hydrocolloid combinations. *Carbohydrate Polymers*, 86(2), 386-423.

Bertoft, E. (2017). Understanding starch structure: Recent progress. *Agronomy*, 7(3), 56.

Bertoft, E., Koch, K., & Åman, P. (2012). Structure of building blocks in amylopectins. *Carbohydrate Research*, 361, 105-113.

Bertoft, E., Piyachomkwan, K., Chatakanonda, P., & Sriroth, K. (2008). Internal unit chain composition in amylopectins. *Carbohydrate Polymers*, 74(3), 527-543.

Bertoft, E. (2004). On the nature of categories of chains in amylopectin and their connection to the super helix model. *Carbohydrate Polymers*, 57(2), 211-224.

Bertoft, E., & Manelius, R. (1992). A method for the study of the enzymatic hydrolysis of starch granules. *Carbohydrate Research*, 227, 269–283.

Betancur, A. D., Chel, G. L., & Cañizares, H. E. (1997). Acetylation and characterization of Canavalia ensiformis starch. *Journal of Agricultural and Food Chemistry*, 45(2), 378-382.

Biliaderis, C. G. (2009). Structural transitions and related physical properties of starch. In *Starch Chemistry and Technology*, J. BeMiller, R. Whistler, Eds., Academic press, NY, pp. 293-359.

Biliaderis, C. G. (1998). Structures and phase transitions of starch polymers. In *Polysaccharide Association Structures in Foods*, R. H. Walter, Ed., Marcel Dekker, New York, NY, pp. 57-168.

Blazek, J., & Gilbert, E. P. (2010). Effect of enzymatic hydrolysis on native starch granule structure. *Biomacromolecules*, 11(12), 3275-3289.

Blennow, A., Hansen, M., Schulz, A., Jørgensen, K., Donald, A. M., & Sanderson, J. (2003). The molecular deposition of transgenically modified starch in the starch granule as imaged by functional microscopy. *Journal of Structural Biology*, 143(3), 229-241.

Blennow, A., Nielsen, T. H., Baunsgaard, L., Mikkelsen, R., & Engelsen, S. B. (2002). Starch phosphorylation: a new front line in starch research. *Trends in Plant Science*, 7(10), 445-450.

Boutboul, A., Giampaoli, P., Feigenbaum, A., & Ducruet, V. (2002). Influence of the nature and treatment of starch on aroma retention. *Carbohydrate Polymers*, 47, 73-82.

Bruner, R.L. (1964). Determination of reducing value. In *Methods in Carbohydrate Chemistry: Starch* (Vol. IV). R.L. Whistler, Ed., Academic Press, NY, pp. 67-71.

Buléon, A. Colonna, P. Planchot V. & Ball, S. (1998). Starch granules: Structure and biosynthesis. *International Journal of Biological Macromolecules*, 23, 85-112.

Buléon, A., Gallant, D. J., Bouchet, B., Mouille, G., D'Hulst, C., Kossmann, J., & Ball, S. (1997). Starches from A to C (chlamydomonasreinhardtii as a model microbial system to investigate the biosynthesis of the plant amylopectin crystal). *Plant Physiology*, 115(3), 949-957.

Burrell, M. M. (2003). Starch: the need for improved quality or quantity—an overview. *Journal of Experimental Botany*, 54(382), 451-456.

Carlson, T. G., Larsson, K., Dinh-Nguyen, N., & Krog, N. (1979). A study of the amylose-monoglyceride complex by raman spectroscopy. *Starch-Stärke*, 31(7), 222-224.

Chao, C., Yu, J., Wang, S., Copeland, L., & Wang, S. (2017). Mechanisms Underlying the Formation of Complexes between Maize Starch and Lipids. *Journal of Agricultural and Food Chemistry*, 66(1), 272-278.

Chauvin, C., Thibault, P., Plusquellec, D., & Banoub, J. (1993). Differentiation of regioisomeric esters of sucrose by ionspray tandem mass spectrometry. *Journal of Carbohydrate Chemistry*, *12*(4-5), 459-475.

Chauhan, F., & Seetharaman, K. (2013). On the organization of chains in amylopectin. *Starch-Stärke*, 65(3-4), 191-199.

Cheetham, N. W., & Tao, L. (1998). Variation in crystalline type with amylose content in maize starch granules: an X-ray powder diffraction study. *Carbohydrate Polymers*, 36(4), 277-284.

Chen, P., Yu, L., Simon, G., Petinakis, E., Dean, K., & Chen, L. (2009). Morphologies and microstructures of cornstarches with different amylose–amylopectin ratios studied by confocal laser scanning microscope. *Journal of Cereal Science*, 50(2), 241-247.

Chen, Z., Schols, H. A., & Voragen, A. G. (2004). Differently sized granules from acetylated potato and sweet potato starches differ in the acetyl substitution pattern of their amylose populations. *Carbohydrate Polymers*, 56(2), 219-226.

Chi, H., Xu, K., Wu, X., Chen, Q., Xue, D., Song, C., Zhang, W., & Wang, P. (2008). Effect of acetylation on the properties of corn starch. *Food Chemistry*, 106(3), 923-928.

Chiang, B. Y., & Johnson, J. A. (1977). Gelatinization of starch in extruded products [Wheat flour]. *Cereal Chemistry (USA)*, 54(3), 436-443.

Chiu, C.W. & Solarek, D. (2009). Modification of starches. In *Starch: Chemistry and Technology*, N.J. BeMiller, R.L. Whistler, Ed., Elsevier Inc, pp 629-655.

Chang, Y., & Lv, Y. (2017). Structure, functionality, and digestibility of acetylated hulless barley starch. *International Journal of Food Properties*, 20(8), 1818-1828.

Chung, H.J., Liu, Q. & Hoover, R. (2010). Effect of single and dual hydrothermal treatments on the crystalline structure, thermal properties, and nutritional fractions of pea, lentil, and navy bean starches. *Food Research International*, 43, 501–508.

Chung, H.J., Hoover, R. & Liu, Q. (2009a). The impact of single and dual hydrothermal modifications on the molecular structure and physicochemical properties of normal corn starch. *International Journal of Biological Macromolecules*, 44, 203–210.

Chung, H.J., Liu, Q. & Hoover, R. (2009b). Impact of annealing and heat-moisture treatment on rapidly digestible, slowly digestible, and resistant starch levels in native and gelatinized corn, pea, and lentil starches. *Carbohydrate Polymers*, 74, 436–447.

Chung, H. J., Shin, D. H., & Lim, S. T. (2008). In vitro starch digestibility and estimated glycemic index of chemically modified corn starches. *Food Research International*, 41(6), 579-585.

Ciucanu, I., & Costello, C. E. (2003). Elimination of oxidative degradation during the per-O-methylation of carbohydrates. *Journal of the American Chemical Society*, 125(52), 16213-16219.

Čmelík, R., & Chmelík, J. (2010). Structural analysis and differentiation of reducing and nonreducing neutral model starch oligosaccharides by negative-ion electrospray ionization ion-trap mass spectrometry. *International Journal of Mass Spectrometry*, 291(1-2), 33-40.

Colonna, P., Leloup, V., & Buleon, A. (1992). Limiting factors of starch hydrolysis. *European Journal of Clinical Nutrition*, 46, S17-32.

Colussi, R., El Halal, S. L. M., Pinto, V. Z., Bartz, J., Gutkoski, L. C., da Rosa Zavareze, E., & Dias, A. R. G. (2015). Acetylation of rice starch in an aqueous medium for use in food. *LWT-Food Science and Technology*, 62(2), 1076-1082.

Colussi, R., Pinto, V. Z., El Halal, S. L. M., Vanier, N. L., Villanova, F. A., e Silva, R. M., Zavareze, E.d.R., & Dias, A. R. G. (2014). Structural, morphological, and physicochemical properties of acetylated high-, medium-, and low-amylose rice starches. *Carbohydrate Polymers*, 103, 405-413.

Cooke, D., & Gidley, M. J. (1992). Loss of crystalline and molecular order during starch gelatinisation: origin of the enthalpic transition. *Carbohydrate Research*, *227*, 103-112.

Copeland, L., Blazek, J., Salman, H., & Tang, M. C. (2009). Form and functionality of starch. *Food Hydrocolloids*, 23(6), 1527-1534.

Craig, S.A.S., Maningat, C.C., Seib, P.A., & Hoseney, R.C. (1989). Starch paste clarity. *Cereal Chemistry*, 66, 173–182.

Crowe, T. C., Seligman, S. A., & Copeland, L. (2000). Inhibition of enzymic digestion of amylose by free fatty acids in vitro contributes to resistant starch formation. *The Journal of Nutrition*, 130(8), 2006-2008.

Cui, R., & Oates, C. G. (1999). The effect of amylose–lipid complex formation on enzyme susceptibility of sago starch. *Food Chemistry*, 65(4), 417-425.

Davis, J. P., Supatcharee, N., Khandelwal, R. L., & Chibbar, R. N. (2003). Synthesis of novel starches in planta: opportunities and challenges. *Starch-Stärke*, 55(3-4), 107-120.

de Graaf, R. A., Broekroelofs, A., & Janssen, L. P. (1998). The acetylation of starch by reactive extrusion. *Starch-Stärke*, 50(5), 198-205.

Debet, M. R., & Gidley, M. J. (2006). Three classes of starch granule swelling: Influence of surface proteins and lipids. *Carbohydrate Polymers*, 64(3), 452-465.

Deetae, P., Shobsngob, S., Varanyanond, W., Chinachoti, P., Naivikul, O., & Varavinit, S. (2008). Preparation, pasting properties and freeze–thaw stability of dual modified cross link-phosphorylated rice starch. *Carbohydrate Polymers*, 73(2), 351-358.

Denyer, K. A. Y., Johnson, P., Zeeman, S., & Smith, A. M. (2001). The control of amylose synthesis. *Journal of Plant Physiology*, 158(4), 479-487.

Dias, A.R.G., Zavareze, E.d.R., Spier, F., de Castro, L.A.S. & Gutkoski, L.C. (2010). Effects of annealing on the physicochemical properties and enzymatic susceptibility of rice starches with different amylose contents. *Food Chemistry*, 123,711–719.

Diop, C. I. K., Li, H. L., Xie, B. J., & Shi, J. (2011). Effects of acetic acid/acetic anhydride ratios on the properties of corn starch acetates. *Food Chemistry*, 126(4), 1662-1669.

Domon, B., & Costello, C. E. (1988). Structure elucidation of glycosphingolipids and gangliosides using high-performance tandem mass spectrometry. *Biochemistry*, 27(5), 1534-1543.

Dona, A. C., Pages, G., Gilbert, R. G., & Kuchel, P. W. (2010). Digestion of starch: In vivo and in vitro kinetic models used to characterise oligosaccharide or glucose release. *Carbohydrate Polymers*, 80(3), 599-617.

Ellis, R. P., Cochrane, M. P., Dale, M. F. B., Duffus, C. M., Lynn, A., Morrison, I. M., Prentice, R.D.K., Swanston, J.S. & Tiller, S. A. (1998). Starch production and industrial use. *Journal of the Science of Food and Agriculture*, 77(3), 289-311.

Emmambux, M. N., & Taylor, J. R. (2013). Morphology, physical, chemical, and functional properties of starches from cereals, legumes, and tubers cultivated in Africa: A review. *Starch-Stärke*, 65(9-10), 715-729.

Englyst, K. N., Englyst, H. N., Hudson, G. J., Cole, T. J., & Cummings, J. H. (1999). Rapidly available glucose in foods: an in vitro measurement that reflects the glycemic response. *American Journal of Clinical Nutrition*, 69, 448-454.

Fannon, J. E., Hauber, R. J., & Bemiller, J. N. (1992). Surface pores of starch granules. *Cereal Chemistry*, 69(3), 284-288.

Flipse, E., Keetels, C. J. A. M., Jacobsen, E., & Visser, R. G. F. (1996). The dosage effect of the wild type GBSS allele is linear for GBSS activity but not for amylose content: absence of amylose has a distinct influence on the physico-chemical properties of starch. *Theoretical and Applied Genetics*, 92(1), 121-127.

Foresti, M. L., del Pilar Williams, M., Martínez-García, R., & Vázquez, A. (2014). Analysis of a preferential action of α-amylase from B. licheniformis towards amorphous regions of waxy maize starch. *Carbohydrate Polymers*, 102, 80-87.

Fredriksson, H., Silverio, J., Andersson, R., Eliasson, A. C., & Åman, P. (1998). The influence of amylose and amylopectin characteristics on gelatinization and retrogradation properties of different starches. *Carbohydrate Polymers*, 35(3-4), 119-134.

Frost, K., Kaminski, D., Kirwan, G., Lascaris, E., & Shanks, R. (2009). Crystallinity and structure of starch using wide angle X-ray scattering. *Carbohydrate Polymers*, 78(3), 543-548.

Fuentes-Zaragoza, E., Riquelme-Navarrete, M.J., Sánchez-Zapata, E. & Pérez-Álvarez, J.A. (2010). Resistant starch as functional ingredient: A review. *Food Research International*, 43, 931–942.

Gallant, D. J., Bouchet, B., & Baldwin, P. M. (1997). Microscopy of starch: evidence of a new level of granule organization. *Carbohydrate Polymers*, 32(3-4), 177-191.

Galliard, T. & Bowler, P. (1987). Morphology and composition of starch. In *Starch, Properties and Potential*, T. Galliard, (Ed), John Wiley, Chichester, UK, pp. 55-78.

Geera, B. P., Nelson, J. E., Souza, E., & Huber, K. C. (2006). Composition and properties of A-and B-type starch granules of wild-type, partial waxy, and waxy soft wheat. *Cereal Chemistry*, 83(5), 551-557.

Geigenberger, P. (2011). Regulation of starch biosynthesis in response to a fluctuating environment. *Plant Physiology*, 155(4), 1566-1577.

Genkina, N. K., Wikman, J., Bertoft, E., & Yuryev, V. P. (2007). Effects of structural imperfection on gelatinization characteristics of amylopectin starches with A-and B-type crystallinity. *Biomacromolecules*, 8(7), 2329-2335.

Genkina, N. K., Noda, T., Koltisheva, G. I., Wasserman, L. A., Tester, R. F., & Yuryev, V. P. (2003). Effects of growth temperature on some structural properties of crystalline lamellae in starches extracted from sweet potatoes (Sunnyred and Ayamurasaki). *Starch-Stärke*, 55(8), 350-357.

Gidley, M. J., & Bulpin, P. V. (1987). Crystallisation of malto-oligosaccharides as models of the crystalline forms of starch: minimum chain-length requirement for the formation of double helices. *Carbohydrate Research*, 161(2), 291-300.

Glaring, M. A., Koch, C. B., & Blennow, A. (2006). Genotype-specific spatial distribution of starch molecules in the starch granule: a combined CLSM and SEM approach. *Biomacromolecules*, 7(8), 2310-2320.

Goesaert, H., Brijs, K., Veraverbeke, W. S., Courtin, C. M., Gebruers, K., & Delcour, J. A. (2005). Wheat flour constituents: how they impact bread quality, and how to impact their functionality. *Trends in Food Science & Technology*, 16(1-3), 12-30.

Golachowski, A., Zięba, T., Kapelko-Żeberska, M., Drożdż, W., Gryszkin, A., & Grzechac, M. (2015). Current research addressing starch acetylation. *Food Chemistry*, 176, 350-356.

Gomand, S,V,, Lamberts, L., Gommes, C.J., Visser, R.G.F., & Delcour, J.A. (2012). Molecular and morphological aspects of annealing-induced stabilization of starch crystallites. *Biomacromolecules*, 13,1361–1370.

Gomand, S. V., Lamberts, L., Visser, R. G. F., & Delcour, J. A. (2010). Physicochemical properties of potato and cassava starches and their mutants in relation to their structural properties. *Food Hydrocolloids*, 24(4), 424-433.

Gray, J. A., & Bemiller, J. N. (2003). Bread staling: molecular basis and control. *Comprehensive Reviews in Food Science and Food Safety*, 2(1), 1-21.

Gunaratne, A., & Corke, H. (2007). Influence of prior acid treatment on acetylation of wheat, potato and maize starches. *Food Chemistry*, *105*(3), 917-925.

Gunaratne, A., & Hoover, R. (2002). Effect of heat–moisture treatment on the structure and physicochemical properties of tuber and root starches. *Carbohydrate Polymers*, 49(4), 425-437.

Gonzalez, Z., & Pérez, E. (2002). Effect of acetylation on some properties of rice starch. *Starch-Stärke*, 54(3-4), 148-154.

Han, F., Liu, M., Gong, H., Lü, S., Ni, B., & Zhang, B. (2012). Synthesis, characterization and functional properties of low substituted acetylated corn starch. *International Journal of Biological Macromolecules*, 50(4), 1026-1034.

Hanashiro, I., Tagawa, M., Shibahara, S., Iwata, K., & Takeda, Y. (2002). Examination of molar-based distribution of A, B and C chains of amylopectin by fluorescent labeling with 2-aminopyridine. *Carbohydrate Research*, 337(13), 1211-1215.

Hanashiro, I., Abe, J. I., & Hizukuri, S. (1996). A periodic distribution of the chain length of amylopectin as revealed by high-performance anion-exchange chromatography. *Carbohydrate Research*, 283, 151-159.

Haralampu, S. G. (2000). Resistant starch—a review of the physical properties and biological impact of RS3. *Carbohydrate Polymers*, 41(3), 285-292.

Harvey, D. J., Bateman, R. H., Bordoli, R. S., & Tyldesley, R. (2000). Ionisation and fragmentation of complex glycans with a quadrupole time-of-flight mass spectrometer fitted with a matrix-assisted laser desorption/ionisation ion source. *Rapid Communications in Mass Spectrometry*, 14(22), 2135-2142.

Hasjim, J., Ai, Y., & Jane, J. L. (2013). Novel applications of amylose-lipid complex as resistant starch type 5. *Resistant Starch: Sources, Applications and Health Benefits*, 79-94.

Heinemann, C., Conde-Petit, B., Nuessli, J., & Escher, F. (2001). Evidence of starch inclusion complexation with lactones. *Journal of Agricultural and Food Chemistry*, 49(3), 1370-1376.

Hernández, O., Emaldi, U., & Tovar, J. (2008). In vitro digestibility of edible films from various starch sources. *Carbohydrate Polymers*, 71, 648–655.

Hizukuri, S. (1993). Towards an understanding of the fine structure of starch molecules. *Journal of the Japanese Society of Starch Science*, 40(2), 133-147.

Hizukuri, S. (1986). Polymodal distribution of the chain lengths of amylopectins, and its significance. *Carbohydrate Research*, 147, 342–347.

Hofmeister, G. E., Zhou, Z., & Leary, J. A. (1991). Linkage position determination in lithium-cationized disaccharides: tandem mass spectrometry and semiempirical calculations. *Journal of the American Chemical Society*, 113(16), 5964-5970.

Hormdok, R., & Noomhorm, A. (2007). Hydrothermal treatments of rice starch for improvement of rice noodle quality. *LWT-Food Science and Technology*, 40(10), 1723-1731.

Hong, J., Zeng, X. A., Buckow, R., & Han, Z. (2018). Structural, thermodynamic and digestible properties of maize starches esterified by conventional and dual methods: Differentiation of amylose contents. *Food Hydrocolloids*, 83, 419-429.

Hong, J. S., Gomand, S. V., & Delcour, J. A. (2015). Preparation of cross-linked maize (Zea mays L.) starch in different reaction media. *Carbohydrate Polymers*, 124, 302-310.

Hoover, R. (2010). The impact of heat-moisture treatment on molecular structures and properties of starches isolated from different botanical sources. *Critical Reviews in Food Science and Nutrition*, 50(9), 835-847.

Hoover, R., Hughes, T., Chung, H. J., & Liu, Q. (2010). Composition, molecular structure, properties, and modification of pulse starches: A review. *Food Research International*, 43(2), 399-413.

Hoover, R. & Ratnayake, W.S. (2004). Determination of total amylose content of starch. In *Handbook of Food Analytical Chemistry - Water, protein, enzymes, lipids, and carbohydrates*, Wrolstad et al., Eds., Wiley-Interscience, Hoboken. NJ, pp. 689-691.

Hoover, R., & Ratnayake, W. S. (2002). Starch characteristics of black bean, chick pea, lentil, navy bean and pinto bean cultivars grown in Canada. *Food Chemistry*, 78(4), 489-498.

Hoover, R. (2001). Composition, molecular structure, and physicochemical properties of tuber and root starches: a review. *Carbohydrate Polymers*, 45(3), 253-267.

Hoover, R. (2000). Acid-treated starches. *Food Reviews International*, 16(3), 369-392.

Hoover, R., & Manuel, H. (1996). The effect of heat–moisture treatment on the structure and physicochemical properties of normal maize, waxy maize, dull waxy maize and amylomaize V starches. *Journal of Cereal Science*, 23(2), 153-162.

Hoover, R., & Manuel, H. (1995). A comparative study of the physicochemical properties of starches from two lentil cultivars. *Food Chemistry*, 53, 275-284.

Hoover, R. (1995). Starch retrogradation. *Food Reviews International*, 11(2), 331-346.

Hoover, R., & Vasanthan, T. (1994). Effect of heat-moisture treatment on the structure and physicochemical properties of cereal, legume, and tuber starches. *Carbohydrate Research*, 252, 33-53.

Hoover, R., & Vasanthan, T. (1993). The effect of annealing on the physicochemical properties of wheat, oat, potato and lentil starches. *Journal of Food Biochemistry*, 17, 303–325.

Hoover, R., & Sosulski, F. (1985). A comparative study of the effect of acetylation on starches of Phaseolus vulgaris biotypes. *Starch-Stärke*, 37(12), 397-404.

Huang, J., Schols, H. A., Jin, Z., Sulmann, E., & Voragen, A. G. (2007). Characterization of differently sized granule fractions of yellow pea, cowpea and chickpea starches after modification with acetic anhydride and vinyl acetate. *Carbohydrate Polymers*, 67(1), 11-20.

Hung, P.V., Chau, H.T. & Phi, N.T.L. (2016). In vitro digestibility and in vivo glucose response of native and physically modified rice starches varying amylose contents. *Food Chemistry*,191, 74-80.

Huang, Y. C., & Lai, H. M. (2014). Characteristics of the starch fine structure and pasting properties of waxy rice during storage. *Food Chemistry*, 152, 432-439.

Imberty, A., Buléon, A., Tran, V., & Péerez, S. (1991). Recent advances in knowledge of starch structure. *Starch-Stärke,* 43(10), 375-384.

Imberty, A., Chanzy, H., Pérez, S., Bulèon, A., & Tran, V. (1988). The double-helical nature of the crystalline part of A-starch. *Journal of Molecular Biology*, 201(2), 365-378.

Jacobs, H., & Delcour, J. A. (1998). Hydrothermal modifications of granular starch, with retention of the granular structure: A review. *Journal of Agricultural and Food Chemistry*, 46(8), 2895-2905.

Jacobs, H., Mischenko, N., Koch, M. H., Eerlingen, R. C., Delcour, J. A., & Reynaers, H. (1998a). Evaluation of the impact of annealing on gelatinisation at intermediate water content of wheat and potato starches: A differential scanning calorimetry and small angle X-ray scattering study. *Carbohydrate Research*, 30*6*(1-2), 1-10.

Jacobs, H., Eerlingen, R. C., Rouseu, N., Colonna, P., & Delcour, J. A. (1998b). Acid hydrolysis of native and annealed wheat, potato and pea starches—DSC melting features and chain length distributions of lintnerised starches. *Carbohydrate Research*, 308(3-4), 359-371.

Jacobs, H., Eerlingen, R. C., Spaepen, H., Grobert, P. J., & Delcour, J. A. (1998c). Impact of annealing on the susceptibility of wheat, potato and pea starches to hydrolysis with pancreatin. *Carbohydrate Research*, 305, 193–207.

Jacobs, H., Eerlingen, R. C., Clauwaert, W., & Delcour, J. A. (1995). Influence of annealing on the pasting properties of starches from varying botanical sources. *Cereal Chemistry*, 72, 480-487.

Jayakody, L., & Hoover, R. (2008). Effect of annealing on the molecular structure and physicochemical properties of starches from different botanical origin – A review. *Carbohydrate Polymers*, 74(3), 691–703.

Jayakody, L., Hoover, R., Liu, Q., & Donner, E. (2007). Studies on tuber starches. II. Molecular structure, composition and physicochemical properties of yam (Dioscorea sp.) starches grown in Sri Lanka. *Carbohydrate Polymers*, 69(1), 148-163.

Jayakody, L., Hoover, R., Liu, Q., & Weber, E. (2005). Studies on tuber and root starches. I. Structure and physicochemical properties of innala (Solenos temonrotundifolius) starches grown in Sri Lanka. *Food Research International*, 38(6), 615-629.

Jayakody, L., & Hoover, R. (2002). The effect of lintnerization on cereal starch granules. *Food Research International*, 35(7), 665-680.

Jane, J. L. (2006). Current understanding on starch granule structures. *Journal of Applied Glycoscience*, 53(3), 205-213.

Jane, J. L., Wong, K. S., & McPherson, A. E. (1997). Branch-structure difference in starches of A-and B-type X-ray patterns revealed by their Naegeli dextrins. *Carbohydrate Research*, 300(3), 219-227.

Jane, J. L., Kasemsuwan, T., Leas, S., Zobel, H., & Robyt, J. F. (1994). Anthology of starch granule morphology by scanning electron microscopy. *Starch-Stärke*, 46(4), 121-129.

Jane, J. L., & Shen, J. J. (1993). Internal structure of the potato starch granule revealed by chemical gelatinization. *Carbohydrate Research*, 247, 279-290.

Jane, J. L., Xu, A., Radosavljevic, M., & Seib, P. A. (1992). Location of amylose in normal starch granules. I. Susceptibility of amylose and amylopectin to cross-linking reagents. *Cereal Chemistry*, 69(4), 405-409.

Jane, J. L., & Robyt, J. F. (1984). Structure studies of amylose-V complexes and retro-graded amylose by action of alpha amylases, and a new method for preparing amylodextrins. *Carbohydrate Research*, 132(1), 105-118.

Jenkins, P. J., & Donald, A. M. (1995). Gelatinisation of starch: a combined SAXS/WAXS/DSC and SANS study. *Carbohydrate Research*, 308(1-2), 133-147.

Jyothi, A.N., Sajeev, M.S. & Sreekumar, J. (2011). Hydrothermal modifications of tropical tuber starches-effect of ANN on the physicochemical, rheological and gelatinization characteristics. *Starch/Stärke*, 63,536-549.

Kailemia, M. J., Ruhaak, L. R., Lebrilla, C. B., & Amster, I. J. (2013). Oligosaccharide analysis by mass spectrometry: a review of recent developments. *Analytical Chemistry*, 86(1), 196-212.

Karas, M., Bahr, U., & Dülcks, T. (2000). Nano-electrospray ionization mass spectrometry: addressing analytical problems beyond routine. *Fresenius' Journal of Analytical Chemistry*, 366(6-7), 669-676.

Karim, A. A., Norziah, M. H., & Seow, C. C. (2000). Methods for the study of starch retrogradation. *Food Chemistry*, 71(1), 9-36.

Kavesh, S., & Schultz, J. M. (1969). Meaning and measurement of crystallinity in polymers: A review. *Polymer Engineering & Science*, 9(5), 331-338.

Kim, B. K., Kim, H. I., Moon, T. W., & Choi, S. J. (2014). Branch chain elongation by amylosucrase: production of waxy corn starch with a slow digestion property. *Food Chemistry*, 152, 113-120.

Kiseleva, V. I., Krivandin, A. V., Fornal, J., Błaszczak, W., Jeliński, T., & Yuryev, V. P. (2005). Annealing of normal and mutant wheat starches. LM, SEM, DSC, and SAXS studies. *Carbohydrate Research*, 340(1), 75-83.

Kiseleva, V. I., Genkina, N. K., Tester, R., Wasserman, L. A., Popov, A. A., & Yuryev, V. P. (2004). Annealing of normal, low and high amylose starches extracted from barley

cultivars grown under different environmental conditions. *Carbohydrate Polymers*, 56, 157–168.

Kong, L., Yucel, U., Yoksan, R., Elias, R. J., & Ziegler, G. R. (2018). Characterization of amylose inclusion complexes using electron paramagnetic resonance spectroscopy. *Food Hydrocolloids*, 82, 82-88.

Lan, H., Hoover, R., Jayakody, L., Liu, Q., Donner, E., Baga, M., Asare, E.K., Hucl, P., & Chibbar, R. N. (2008). Impact of annealing on the molecular structure and physicochemical properties of normal, waxy and high amylose bread wheat starches. *Food Chemistry*, 111(3), 663-675.

Lawal, O. S., & Adebowale, K. O. (2005). Physicochemical characteristics and thermal properties of chemically modified jack bean (Canavalia ensiformis) starch. *Carbohydrate Polymers*, 60(3), 331-341.

Le Corre, D., Bras, J., & Dufresne, A. (2010). Starch nanoparticles: a review. *Biomacromolecules*, 11(5), 1139-1153.

Lee, C.J. & Moon, T.W. (2015). Structural characteristics of slowly digestible starch and resistant starch isolated from heat-moisture treated waxy potato starch. *Carbohydrate Polymers*,125, 200-205.

Leach, W., McCowen, D. & Schoch, T. J. (1959). Structure of the starch granule. I. Swelling and solubility patterns of various starches. *Cereal Chemistry*, 36, 534-544.

Lehmann, U., & Robin, F. (2007). Slowly digestible starch–its structure and health implications: a review. *Trends in Food Science & Technology*, 18(7), 346-355.

Leloup, V. M., Colonna, P., & Ring, S. G. (1990). Studies on probe diffusion and accessibility in amylose gels. *Macromolecules*, 23(3), 862-866.

Leszczynski, W. (2004). Resistant starch-classification, structure, production. *Polish Journal of Food and Nutrition Sciences.* 13(54), 37-55.

Li, J. H., Vasanthan, T., Hoover, R., & Rossnagel, B. G. (2004). Starch from hull-less barley: V. In-vitro susceptibility of waxy, normal, and high-amylose starches towards hydrolysis by alpha-amylases and amyloglucosidase. *Food Chemistry*, 84(4), 621-632.

Li, J. Y., & Yeh, A. I. (2001). Relationships between thermal, rheological characteristics and swelling power for various starches. *Journal of Food Engineering*, 50(3), 141-148.

Lim, S. T., Kasemsuwan, T. & Jane, J. (1994). Characterization of phosphorus in starch by 31P-nuclear magnetic resonance spectroscopy. *Cereal Chemistry.*, 71, 488-493.

Lim, S., & Seib, P. A. (1993). Preparation and pasting properties of wheat and corn starch phosphates. *Cereal Chemistry*, 70, 137-137.

Lindeboom, N., Chang, P. R., & Tyler, R. T. (2004). Analytical, biochemical and physicochemical aspects of starch granule size, with emphasis on small granule starches: a review. *Starch-Stärke*, 56(3-4), 89-99.

Liu, H., Yu, L., Simon, G., Dean, K., & Chen, L. (2009). Effects of annealing on gelatinization and microstructures of corn starches with different amylose/amylopectin ratios. *Carbohydrate Polymers*, 77,662–669.

Liu, Q., Gu, Z., Donner, E., Tetlow, I., & Emes, M. (2007). Investigation of digestibility in vitro and physicochemical properties of A-and B-type starch from soft and hard wheat flour. *Cereal Chemistry*, 84(1), 15–21.

Liu, Q., & Thompson, D. B. (1998). Effects of moisture content and different gelatinization heating temperatures on retrogradation of waxy-type maize starches. *Carbohydrate Research*, 314(3-4), 221-235.

Liu, H., Ramsden, L., & Corke, H. (1997). Physical properties and enzymatic digestibility of acetylated ae, wx, and normal maize starch. *Carbohydrate Polymers*, 34(4), 283-289.

Longton, J., & LeGrys, G. A. (1981). Differential scanning calorimetry studies on the crystallinity of ageing wheat starch gels. *Starch-Stärke*, 33(12), 410-414.

Lorberth, R., Ritte, G., Willmitzer, L., & Kossmann, J. (1998). Inhibition of a starch-granule–bound protein leads to modified starch and repression of cold sweetening. *Nature Biotechnology*, 16(5), 473.

Lopez-Rubio, A., Flanagan, B. M., Gilbert, E. P., & Gidley, M. J. (2008). A novel approach for calculating starch crystallinity and its correlation with double helix content: A combined XRD and NMR study. *Biopolymers: Original Research on Biomolecules*, 89(9), 761-768.

Lu, D., Cai, X., Yan, F., Sun, X., Wang, X., & Lu, W. (2014). Effects of high temperature after pollination on physicochemical properties of waxy maize flour during grain development. *Journal of the Science of Food and Agriculture*, 94(7), 1416-1421.

Lu, S., Chen, L. N., & Lii, C. Y. (1997). Correlations between the fine structure, physicochemical properties, and retrogradation of amylopectins from Taiwan rice varieties. *Cereal Chemistry*, 74(1), 34-39.

Luo, Z. G., & Shi, Y. C. (2012). Preparation of acetylated waxy, normal, and high-amylose maize starches with intermediate degrees of substitution in aqueous solution and their properties. *Journal of Agricultural and Food Chemistry*, 60(37), 9468-9475.

Martin, C., & Smith, A. M. (1995). Starch biosynthesis. *The Plant Cell*, 7(7), 971.

Mbougueng, P. D., Tenin, D., Scher, J., & Tchiégang, C. (2012). Influence of acetylation on physicochemical, functional and thermal properties of potato and cassava starches. *Journal of Food Engineering*, 108(2), 320-326.

McLafferty, F. W., & Turecek, F. (1993). *Interpretation of Mass Spectra*. University Science Books, Mill Valley, CA.

McPherson, A. E., & Jane, J. L. (1999). Comparison of waxy potato with other root and tuber starches. *Carbohydrate Polymers*, 40(1), 57-70.

Mischnick, P. (2011). Mass spectrometric characterization of oligo-and polysaccharides and their derivatives. In *Mass Spectrometry of Polymers–New Techniques*, M. Hakkarainen, Ed., Springer, Berlin, Heidelberg. pp. 105-174.

Miyazaki, M., Van Hung, P., Maeda, T., & Morita, N. (2006). Recent advances in application of modified starches for breadmaking. *Trends in Food Science & Technology*, 17(11), 591-599.

Morrison, W. R., Law, R. V., & Snape, C. E. (1993). Evidence for inclusion complexes of lipids with V-amylose in maize, rice and oat starches. *Journal of Cereal Science*, 18(2), 107-109.

Morrison, W. R., & Karkalas, J. (1990). Starch. In *Methods in plant Biochemistry:Carbohydrates*, P.M. Dey, Ed., Academic Press, London, UK, pp. 252-323.

Morrison, W. R., Milligan, T. P., & Azudin, M. N. (1984). A relationship between the amylose and lipid contents of starches from diploid cereals. *Journal of Cereal Science*, 2(4), 257-271.

Muhrbeck, P., & Eliasson, A. C. (1991). Influence of the naturally occurring phosphate esters on the crystallinity of potato starch. *Journal of the Science of Food and Agriculture*, 55(1), 13-18.

Muhrbeck, P., & Svensson, E. (1996). Annealing properties of potato starches with different degrees of phosphorylation. *Carbohydrate Polymers*, 31(4), 263-267.

Naguleswaran, S., Vasanthan, T., Hoover, R., & Bressler, D. (2013). The susceptibility of large and small granules of waxy, normal and high-amylose genotypes of barley and corn starches toward amylolysis at sub-gelatinization temperatures. *Food Research International*, 51(2), 771-782.

Nakazawa, Y., & Wang, Y. J. (2003). Acid hydrolysis of native and annealed starches and branch-structure of their Naegeli dextrins. *Carbohydrate Research*, 338(24), 2871-2882.

Neelam, K., Vijay, S., & Lalit, S. (2012). Various techniques for the modification of starch and the applications of its derivatives. *International Research Journal of Pharmacy*, *3*(5), 25-31.

Niba, L. L. (2002). Resistant starch: a potential functional food ingredient. *Nutrition & Food Science*, 32(2), 62-67.

Norisuye, T. (1996). Conformation and properties of amylose in dilute solution. *Food Hydrocolloids*, 10(1), 109-115.

Nugent, A. P. (2005). Health properties of resistant starch. British Nutrition Foundation, *Nutrition Bulletin*, 30, 27–54.

O'Brien & Wang, J. Y. (2008). Susceptibility of annealed starches to hydrolysis by α amylase and glucoamylase. *Carbohydrate Polymers*, 72, 597–607.

Orford, P. D., Ring, S. G., Carroll, V., Miles, M. J., & Morris, V. J. (1987). The effect of concentration and botanical source on the gelation and retrogradation of starch. *Journal of the Science of Food and Agriculture*, 39(2), 169-177.

Osundahunsi, O. F., & Mueller, R. (2011). Functional and dynamic rheological properties of acetylated starches from two cultivars of cassava. *Starch-Stärke*, 63(1), 3-10.

Ottenhof, M.-A., & Farhat, I. A. (2004). Starch retrogradation. *Biotechnology and Genetic Engineering Reviews*, 21(1), 215-228.

Ozcan, S., & Jackson, D. S. (2002). The impact of thermal events on amylose-fatty acid complexes. *Starch-Stärke*, 54(12), 593-602.

Peat, S., Whelan, W. J., & Thomas, G. J. (1956). 587. The enzymic synthesis and degradation of starch. Part XXII. Evidence of multiple branching in waxy-maize starch. A correction. *Journal of the Chemical Society (Resumed)*, 3025-3030

Peat, S., Whelan, W. J., & Thomas, G. J. (1952). Evidence of multiple branching in waxy maize starch. *Journal of the Chemical Society Chemical Communications*, 4546-4548.

Perera, A., Meda, V. & Tyler, R.T. (2010). Resistant starch: A review of analytical protocols for determining resistant starch and of factors affecting the resistant starch content of foods. *Food Research International*, 43, 1959-1974.

Perera, C., & Hoover, R. (1999). Influence of hydroxypropylation on retrogradation properties of native, defatted and heat-moisture treated potato starches. *Food Chemistry*, 64(3), 361-375.

Pérez, S., & Bertoft, E. (2010). The molecular structures of starch components and their contribution to the architecture of starch granules: A comprehensive review. *Starch-Stärke*, 62(8), 389-420.

Pfannemüller, B. (1987). Influence of chain length of short monodisperse amyloses on the formation of A-and B-type X-ray diffraction patterns. *International Journal of Biological Macromolecules*, 9(2), 105-108.

Phillips, L. D., Liu, H., Pan, D., & Corke, H. (1999). General application of Raman spectroscopy for the determination of level of acetylation in modified starches. *Cereal Chemistry*, 76(3), 439-443.

Polce, M. J., & Westdemiotis, C. (2002) Introduction to Mass Spectrometry of polymers. In *Mass Spectrometry of Polymers,*G. Montaudo, R.P. Lattimer, Eds., CRC Press, Florida, pp 1-40.

Putseys, J. A., Lamberts, L., & Delcour, J. A. (2010). Amylose-inclusion complexes: Formation, identity and physico-chemical properties. *Journal of Cereal Science*, 51(3), 238-247.

Raghunathan, R., Hoover, R., Waduge, R., Liu, Q., & Warkentin, T. D. (2017). Impact of molecular structure on the physicochemical properties of starches isolated from different field pea (Pisumsativum L.) cultivars grown in Saskatchewan, Canada. *Food Chemistry*, 221, 1514-1521.

Ratnayake, W. S., & Jackson, D. S. (2007). Starch gelatinization. *Advances in Food and Nutrition Research*, 55, 221-268.

Ratnayake, W. S., Hoover, R., Shahidi, F., Perera, C., & Jane, J. (2001). Composition, molecular structure, and physicochemical properties of starches from four field pea (Pisum sativum L.) cultivars. *Food Chemistry*, 74(2), 189-202.

Ring, S. G., Colonna, P., I'Anson, K. J., Kalichevsky, M. T., Miles, M. J., Morris, V. J., & Orford, P. D. (1987). The gelation and crystallisation of amylopectin. *Carbohydrate Research*, *162*(2), 277-293.

Ritte, G., Lloyd, J. R., Eckermann, N., Rottmann, A., Kossmann, J., & Steup, M. (2002). The starch-related R1 protein is an α-glucan, water dikinase. *Proceedings of the National Academy of Sciences*, 99(10), 7166-7171.

Robin, J. P., Mercier, C., Charbonniere, R., & Guilbot, A. (1974). Lintnerized starches. Gel-filtration and enzymatic studies of insoluble residues from prolonged acid treatment of potato starch. *Cereal Chemistry*, 51, 389-406.

Rocha, T. S., Gelizardo, S. G., Jane, J.-L., & Franco, C. M. L. (2012). Effect of annealing on the semicrystalline structure of normal and waxy maize starches. *Food Hydrocolloids*, 29(1), 93–99.

Rocha, T. S., Cunha, V. A., Jane, J. L., & Franco, C. M. (2011). Structural characterization of Peruvian carrot (Arracacia xanthorrhiza) starch and the effect of annealing on its semicrystalline structure. *Journal of Agricultural and Food Chemistry*, 59(8), 4208-4216.

Ruhaak, L. R., Zauner, G., Huhn, C., Bruggink, C., Deelder, A. M., & Wuhrer, M. (2010). Glycan labeling strategies and their use in identification and quantification. *Analytical and Bioanalytical Chemistry*, 397(8), 3457-3481.

Saibene, D., & Seetharaman, K. (2010). Amylose involvement in the amylopectin clusters of potato starch granules. *Carbohydrate Polymers*, 82(2), 376-383.

Sajilata, M. G., Singhal, R. S., & Kulkarni, P. R. (2006). Resistant starch–a review. *Comprehensive Reviews in Food Science and Food Safety*, 5(1), 1-17.

Sakonidou, E. P., Karapantsios, T. D., & Raphaelides, S. N. (2003). Mass transfer limitations during starch gelatinization. *Carbohydrate Polymers*, 53(1), 53-61.

Šárka, E., & Dvořáček, V. (2017). Waxy starch as a perspective raw material (a review). *Food Hydrocolloids*, 69, 402-409.

Sarko, A., & Wu, H. C. (1978). The crystal structures of A-, B-and C-polymorphs of amylose and starch. *Starch-Stärke*, 30(3), 73-78.

Sasaki, T., & Matsuki, J. (1998). Effect of wheat starch structure on swelling power. *Cereal Chemistry*, 75(4), 525-529.

Senior, M. B., & Hamori, E. (1973). Investigation of the effect of amylose/iodine complexation on the conformation of amylose in aqueous solution. *Biopolymers: Original Research on Biomolecules*, 12(1), 65-78.

Shi, Y. C. (2008). Two-and multi-step annealing of cereal starches in relation to gelatinization. *Journal of Agricultural and Food Chemistry*, 56(3), 1097-1104.

Shi, Y. C., Capitani, T., Trzasko, P., & Jeffcoat, R. (1998). Molecular structure of a low-amylopectin starch and other high-amylose maize starches. *Journal of Cereal Science*, 27(3), 289-299.

Shi, Y. C., & Seib, P. A. (1992). The structure of four waxy starches related to gelatinization and retrogradation. *Carbohydrate Research*, 227, 131-145.

Silverio, J., Fredriksson, H., Andersson, R., Eliasson, A. C., & Åman, P. (2000). The effect of temperature cycling on the amylopectin retrogradation of starches with different amylopectin unit-chain length distribution. *Carbohydrate Polymers*, 42(2), 175-184.

Silverio, J., Svensson, E., Eliasson, A. C., & Olofsson, G. (1996). Isothermal microcalorimetric studies on starch retrogradation. *Journal of Thermal Analysis and Calorimetry*, 47(5), 1179-1200.

Simsek, S., Ovando-Martínez, M., Whitney, K., & Bello-Pérez, L. A. (2012). Effect of acetylation, oxidation and annealing on physicochemical properties of bean starch. *Food Chemistry*, 134(4), 1796-1803.

Singh, H., Sodhi, N. S., & Singh, N. (2012). Structure and functional properties of acetylated sorghum starch. *International Journal of Food Properties*, 15(2), 312-325.

Singh, J., Dartois, A. & Kaur, L. (2010). Starch digestibility in food matrix: a review.*Trends in Food Science & Technology*, 21,168-180.

Singh, A. V., Nath, L. K., & Singh, A. (2010). Pharmaceutical, food and non-food applications of modified starches: a critical review.*Electronic Journal of Environmental, Agricultural and Food Chemistry* , 9(7), 1214-1221.

Singh, N., Chawla, D., & Singh, J. (2004). Influence of acetic anhydride on physicochemical, morphological and thermal properties of corn and potato starch. *Food Chemistry*, 86(4), 601-608.

Singh, N., Singh, J., Kaur, L., Sodhi, N. S., & Gill, B. S. (2003). Morphological, thermal and rheological properties of starches from different botanical sources. *Food Chemistry*, 81(2), 219-231.

Slade, L., & Levine, H. (1988). Non-equilibrium behavior of small carbohydrate-water systems. *Pure and Applied Chemistry*, 60(12), 1841-1864.

Slade, L., & Levine, H. (1987). Recent advances in starch retrogradation. *Industrial Polysaccharides*, 387-430.

Sodhi, N. S., & Singh, N. (2005). Characteristics of acetylated starches prepared using starches separated from different rice cultivars. *Journal of Food Engineering*, *70*(1), 117-127.

Sodhi N.S., & Singh N. (2003). Morphological, thermal and rheological properties of starches separated from rice cultivars grown in India. *Food Chemistry*, 80, 99–108.

Song, Y., & Jane, J. L. (2000). Characterization of barley starches of waxy, normal, and high amylose varieties. *Carbohydrate Polymers*, 41(4), 365-377.

Srikaeo, K. (2016). Starch: Introduction and structure-property relationship. In *Starch-based Blends, Composites and Nanocomposites,* P.M. Visakh, L.Yu, Eds., London, UK: Royal Society of Chemistry, pp 17-59.

Stevens, D. J., & Elton, G. A. H. (1971). Thermal properties of the starch/water system Part I. measurement of heat of gelatinisation by differential scanning calorimetry. *Starch-Stärke*, 23(1), 8-11.

Sujka, M., & Jamroz, J. (2007). Starch granule porosity and its changes by means of amylolysis. *International Agrophysics*, 21(1), 107.

Sun, S., Zhang, G., & Ma, C. (2016). Preparation, physicochemical characterization and application of acetylated lotus rhizome starches. *Carbohydrate Polymers,* 135, 10-17.

Tahir, R., Ellis, P. R., Bogracheva, T. Y., Meares-Taylor, C., & Butterworth, P. J. (2010). Study of the structure and properties of native and hydrothermally processed wild-type, lam and r variant pea starches that affect amylolysis of these starches. *Biomacromolecules*, 12(1), 123-133.

Takeda, Y., Shibahara, S., & Hanashiro, I. (2003). Examination of the structure of amylopectin molecules by fluorescent labeling. *Carbohydrate Research*, 338(5), 471-475.

Takeda, Y., Maruta, N., & Hizukuri, S. (1992). Structures of amylose subfractions with different molecular sizes. *Carbohydrate Research*, 226(2), 279-285.

Takeda, Y., Hizukuri, S., Takeda, C., & Suzuki, A. (1987). Structures of branched molecules of amyloses of various origins, and molecular fractions of branched and unbranched molecules. *Carbohydrate Research*, 164, 139–145.

Tang, M. C., & Copeland, L. (2007). Investigation of starch retrogradation using atomic force microscopy. *Carbohydrate Polymers*, 70(1), 1-7.

Tatongjai, J., & Lumdubwong, N. (2010). Physicochemical properties and textile utilization of low-and moderate-substituted carboxymethyl rice starches with various amylose content. *Carbohydrate Polymers*, 81(2), 377-384.

Tao, H., Wang, P., Wu, F., Jin, Z., & Xu, X. (2016). Particle size distribution of wheat starch granules in relation to baking properties of frozen dough. *Carbohydrate Polymers*, 137, 147-153.

Tetlow, I. J., Wait, R., Lu, Z., Akkasaeng, R., Bowsher, C. G., Esposito, S., Kosar-Hashemi, B., Morell, M. K., & Emes, M. J. (2004). Protein phosphorylation in amyloplasts regulates starch branching enzyme activity and protein–protein interactions. *The Plant Cell*, 16(3), 694-708.

Tester, R. F., Karkalas, J., & Qi, X. (2004). Starch—composition, fine structure and architecture. *Journal of Cereal Science*, 39(2), 151-165.

Tester, R. F., & Karkalas, J. (2002). Starch. In *Biopolymers Volume 6, Polysaccharides II: Polysaccharides from Eukaryotes*, A. Steinbuchel, series Ed., E. J.Vandamme, S. De Baets and A. Steinbtichel, volume Eds, Wiley-VCH,Weinheim, pp. 381-438.

Tester, R. F., Debon, S. J. J., & Sommerville, M. D. (2000). Annealing of maize starch. *Carbohydrate Polymers*, 42, 287-299.

Tester, R. F., & Debon, S. J. (2000). Annealing of starch—a review. *International Journal of Biological Macromolecules*, 27(1), 1-12.

Tester, R. F., Debon, S. J. J., Davies, H. V., & Gidley, M. J. (1999). Effect of temperatureon the synthesis, composition and physical properties of potato starch. *Journal of the Science of Food and Agriculture*, 79, 2045-2051.

Tester, R. F., Debon, S. J. J. & Karkalas, J. (1998). Annealing of wheat starch. *Journal of Cereal Science*, 28, 259–72.

Tester, R. F. (1997). Influence of growth conditions on barley starch properties. *International Journal of Biological Macromolecules*, 21(1-2), 37-45.

Tester, R. F., & Morrison, W. R. (1990a). Swelling and gelatinization of cereal starches. I. Effects of amylopectin, amylose, and lipids. *Cereal Chemistry*, 67(6), 551-557.

Tester, R. F., & Morrison, W. R. (1990b). Swelling and gelatinization of cereal starches. II. Waxy rice starches. *Cereal Chemistry*, 67(6), 558-563.

Thygesen, L. G., Blennow, A., & Engelsen, S. B. (2003). The effects of amylose and starch phosphate on starch gel retrogradation studied by low-field 1H NMR relaxometry. *Starch-Stärke*, 55(6), 241-249.

Tomlinson, K., & Denyer, K. (2003). Starch synthesis in cereal grains. *Advances in Botanical Research*, 40, 1-61.

Tran, U. T., Okadome, H., Murata, M., Homma, S., & Ohtsubo, K. I. (2001). Comparison of Vietnamese and Japanese rice cultivars in terms of physicochemical properties. *Food Science and Technology Research*, 7(4), 323-330.

Uthumporn, U., Zaidul, I. S., & Karim, A. A. (2010). Hydrolysis of granular starch at sub-gelatinization temperature using a mixture of amylolytic enzymes. *Food and Bioproducts Processing*, 88(1), 47-54.

Vamadevan, V., & Bertoft, E. (2015). Structure-function relationships of starch components. *Starch-Stärke*, 67(1-2), 55-68.

Vamadevan, V., & Bertoft, E. (2014). Structure-function relationships of starch components. *Starch/Stärke*, 66, 1-14.

Vamadevan, V., Bertoft, E., & Seetharaman, K. (2013a). On the importance of organization of glucan chains on thermal properties of starch. *Carbohydrate Polymers*, 92(2), 1653-1659.

Vamadevan, V., Bertoft, E., Soldatov, D.V. & Seetharaman, K. (2013b). Impact on molecular organization of amylopectin in starch granules upon annealing. *Carbohydrate Polymers*, 98,1045–1055.

Van Der Maarel, M. J., Van der Veen, B., Uitdehaag, J. C., Leemhuis, H., & Dijkhuizen, L. (2002). Properties and applications of starch-converting enzymes of the α-amylase family. *Journal of Biotechnology*, 94(2), 137-155.

van der Burgt, Y. E., Bergsma, J., Bleeker, I. P., Mijland, P. J., van der Kerk-van Hoof, A., Kamerling, J. P., & Vliegenthart, J. F. (2000). Distribution of methyl substituents in amylose and amylopectin from methylated potato starches. *Carbohydrate Research*, 325(3), 183-191.

van der Burgt, Y. E., Bergsma, J., Bleeker, I. P., Mijland, P. J., van der Kerk-van Hoof, A., Kamerling, J. P., & Vliegenthart, J. F. (1999). Distribution of methyl substituents over crystalline and amorphous domains in methylated starches. *Carbohydrate Research*, 320(1-2), 100-107.

van der Burgt, Y. E., Bergsma, J., Bleeker, I. P., Mijland, P. J., van der Kerk-van Hoof, A., Kamerling, J. P., & Vliegenthart, J. F. (1998). Distribution of methyl substituents over branched and linear regions in methylated starches. *Carbohydrate Research*, 312(4), 201-208.

Varatharajan, V., Hoover, R., Liu, Q., & Seetharaman, K. (2010). The impact of heat-moisture treatment on the molecular structure and physicochemical properties of normal and waxy potato starches. *Carbohydrate Polymers*, 81(2), 466-475.

Varavinit, S., Paisanjit, W., Tukomane, T., & Pukkahuta, C. (2007). Effects of osmotic pressure on the cross linking reaction of tapioca starch. *Starch-Stärke*, 59(6), 290-296.

Vasanthan, T., & Bhatty, R. S. (1996). Physicochemical properties of small-and large-granule starches of waxy, regular, and high-amylose barleys. *Cereal Chemistry*, 73(2), 199-207.

Viksø-Nielsen, A., Blennow, A., Jørgensen, K., Kristensen, K. H., Jensen, A., & Møller, B. L. (2001). Structural, Physicochemical, and pasting properties of starches from potato plants with repressed r 1-gene. *Biomacromolecules*, 2(3), 836-843.

Waduge, R. N., Hoover, R., Vasanthan, T., Gao, J., & Li, J. (2006). Effect of annealing on the structure and physicochemical properties of barley starches of varying amylose content. *Food Research International*, 39(1), 59-77.

Waigh, T. A., Gidley, M. J., Komanshek, B. U., & Donald, A. M. (2000). The phase transformations in starch during gelatinisation: a liquid crystalline approach. *Carbohydrate Research*, 328(2), 165-176.

Wang, S., & Copeland, L. (2015). Effect of acid hydrolysis on starch structure and functionality: A review. *Critical Reviews in Food Science and Nutrition*, 55(8), 1081-1097.

Wang, S., Li, C., Copeland, L., Niu, Q., & Wang, S. (2015). Starch retrogradation: A comprehensive review. *Comprehensive Reviews in Food Science and Food Safety*, 14(5), 568-585.

Wang, S., Wang, J., Yu, J., & Wang, S. (2014). A comparative study of annealing of waxy, normal and high-amylose maize starches: The role of amylose molecules. *Food Chemistry*, 164, 332-338.

Wang, S., & Copeland, L. (2013). Molecular disassembly of starch granules during gelatinization and its effect on starch digestibility: a review. *Food & Function*, 4(11), 1564-1580.

Wang, Y. J., & Wang, L. (2002). Characterization of acetylated waxy maize starches prepared under catalysis by different alkali and alkaline-earth hydroxides. *Starch-Stärke,* 54(1), 25-30.

Wang, J., Jiang, G., Vasanthan, T., & Sporns, P. (1999). MALDI-MS characterization of maltooligo/polysaccharides from debranched starch amylopectin of corn and barley. *Starch-Stärke,* 51(7), 243-248.

Wang, L. Z. & White, P. J. (1994). Structure and properties of amylose, amylopectin and intermediate material of oat starches. *Cereal Chemistry,* 71, 263-268.

Wani, A. A., Singh, P., Shah, M. A., Schweiggert-Weisz, U., Gul, K., & Wani, I. A. (2012). Rice starch diversity: Effects on structural, morphological, thermal, and physicochemical properties—A review. *Comprehensive Reviews in Food Science and Food Safety,* 11(5), 417-436.

Ward, K. E. J., Hoseney, R. C., & Seib, P. A. (1994). Retrogradation of amylopectin from maize and wheat starches. *Cereal Chemistry,* 71(2), 150-154.

Whittam, M. A., Orford, P. D., Ring, S. G., Clark, S. A., Parker, M. L., Cairns, P., & Miles, M. J. (1989). Aqueous dissolution of crystalline and amorphous amylose-alcohol complexes. *International Journal of Biological Macromolecules,* 11(6), 339-344.

Whistler, R. L., & Daniel, J. R. (1995). Carbohydrates. In *Food Chemistry,* O. R. Fennema Ed., New York: Marcel Decker, pp. 69-137.

Wiesenborn, d. P., Orr, P. H., Casper, H. H., & Tacke, B. K. (1994). Potato starch paste behavior as related to some physical/chemical properties. *Journal of Food Science,* 59(3), 644-648.

Wootton, M., & Bamunuarachchi, A. (1979). Application of differential scanning calorimetry to starch gelatinization. I. Commercial native and modified starches. *Starch-Stärke*, 31(6), 201-204.

Wu, H.C.H., & Sarko, A. (1978a). The double-helical molecular structure of crystalline B-amylose. *Carbohydrate Research*, 61(1), 7-25.

Wu, H.C.H., & Sarko, A. (1978b). The double-helical molecular structure of crystalline A-amylose. *Carbohydrate Research*, 61(1), 27-40

Wuhrer, M. (2013). Glycomics using mass spectrometry. *Glycoconjugate Journal*, 30(1), 11-22.

Würsch, P., & Gumy, D. (1994). Inhibition of amylopectin retrogradation by partial beta-amylolysis. *Carbohydrate Research*, 256(1), 129-137.

Wurzburg, O. B. (1995). Modified starches. In *Food Polysaccharides and Their Applications* A. M. Stephen, Ed., New York: Marcel Decker, pp. 83–85.

Wurzburg, O. B. (1986). *Modified starches: Properties and uses*. Boca Raton, FL: CRC Press.

Wurzburg, O. B. (1964). Acetylation. In *Methods in Carbohydrate Chemistry*, R. L. Whistler Ed., Boca Raton, FL: Academic Press, p. 240.

Xia, L., Wenyuan, G., Qianqian, J., Luqi, H., & Changxiao, L. (2011). Study on the morphology, crystalline structure, and thermal properties of Fritillaria ussuriensis Maxim. starch acetates with different degrees of substitution. *Starch-Stärke*, 63(1), 24-31.

Xie, Y. Y., Hu, X. P., Jin, Z. Y., Xu, X. M., & Chen, H. Q. (2014). Effect of repeated retrogradation on structural characteristics and in vitro digestibility of waxy potato starch. *Food chemistry*, 163, 219-225.

Xu, S. S., Xiang, Z. J., Bin, L., Jing, L., Bin, Z., Jiao, Y. J., & Kun, S. R. (2012). Preparation and physical characteristics of resistant starch (type 4) in acetylated indica rice. *Food Chemistry*, 134(1), 149-154.

Xu, Y., Miladinov, V., & Hanna, M. A. (2004). Synthesis and characterization of starch acetates with high substitution. *Cereal Chemistry*, 81(6), 735-740.

Yao, Y., Guiltinan, M. J., & Thompson, D. B. (2005). High-performance size-exclusion chromatography (HPSEC) and fluorophore-assisted carbohydrate electrophoresis (FACE) to describe the chain-length distribution of debranched starch. *Carbohydrate Research*, 340(4), 701-710.

Yoshida, H., Nozaki, K., Hanashiro, 1., Yagi, F., Ito, H., Honrna, M., Matsui, H., & Takeda, Y. (2003). Structure and physicochemical properties of starches from kidney bean seeds at immature, premature and mature stages of development, *Carbohydrate Research*, 38, 463-469.

Yoshimoto, Y., Tashiro, J ., Takenouchi, T.,& Takeda, Y. (2000). Molecular structureand some physicochemical properties of high-amylose barley starch. *Cereal Chemistry*, 77, 279-285.

Zaia, J. (2004). Mass spectrometry of oligosaccharides. *Mass Spectrometry Reviews*, 23(3), 161-227.

Zavareze, E.d.R & Dias, A.R.G. (2011). Impact of heat-moisture treatment and annealing in starches: a review. *Carbohydrate Polymers*, 83, 317–328.

Zeleznak, K. J., & Hoseney, R. C. (1986). The role of water in the retrogradation of wheat starch gels and bread crumb. *Cereal Chemistry*, 63(5), 407-411.

Zeeman, S. C., Kossmann, J., & Smith, A. M. (2010). Starch: its metabolism, evolution, and biotechnological modification in plants. *Annual Review of Plant Biology*, 61, 209-234.

Zeng, F., Ma, F., Kong, F., Gao, Q. & Yu, S. (2015). Physicochemical properties and digestibility of hydrothermally treated waxy rice starch. *Food Chemistry*, 172, 92-98.

Zhang, H., Zhang, W., Xu, C., & Zhou, X. (2014). Studies on the rheological and gelatinization characteristics of waxy wheat flour. *International Journal of Biological Macromolecules*, 64, 123-129.

Zhang, B., Dhital, S., & Gidley, M. J. (2013). Synergistic and antagonistic effects of α-amylase and amyloglucosidase on starch digestion. *Biomacromolecules*, 14(6), 1945-1954.

Zhang, G. & Hamaker, B.R (2009). Slowly digestible starch: concept, mechanism, and proposed extended glycemic index. *Critical Reviews in Food Science and Nutrition*, 49, 852-867.

Zhang, G., Ao, Z. & Hamaker, B. R. (2006a). Slow digestion property of native cereal starches. *Biomacromolecules*, 7, 3252–3258.

Zhang, G., Venkatachalam, M. & Hamaker, B. R. (2006b). Structural basis for the slow digestion property of native cereal starch. *Biomacromolecules*, 7,3259–3266.

Zhou, Z., Ogden, S., & Leary, J. A. (1990). Linkage position determination in oligosaccharides: mass spectrometry (MS/MS) study of lithium-cationized carbohydrates. *The Journal of Organic Chemistry*, 55(20), 5444-5446.

Zięba, T., Szumny, A., & Kapelko, M. (2011). Properties of retrograded and acetylated starch preparations: Part 1. Structure, susceptibility to amylase, and pasting characteristics. *LWT-Food Science and Technology*, 44(5), 1314-1320.

Zobel, H. F. (1988). Molecules to granules: a comprehensive starch review. *Starch-Stärke*, 40(2), 44-50.

**Publications**

Impact of annealing on the hierarchical structure and physicochemical properties of waxy starches of different botanical origins, *Food Chemistry* (under review).

**Conferences/ Presentations**

1. Egodage R.J.S., Hoover R., Shahidi F., Banoub J.H. (2018). Impact of annealing, acetylation, and dual modification on *in vitro* digestibility and nutritional fractions ofwaxy starches. *The International Society for Nutraceuticals and Functional Foods (ISNFF),* Vancouver, BC, Canada.
2. Egodage R.J.S., Hoover R., Shahidi F., Banoub J.H. (2018). Impact of chemical modification on structure and properties of waxy starches. *8th Biochemistry Summer Symposium,* St. John's, NL, Canada.
3. Egodage R.J.S., Hoover R., Shahidi F., Banoub J.H. (2018). Impact of chemical modification on the structure and properties of waxy starches of different botanical origins. *IFT18 Annual Meeting & Food Expo,* Chicago, IL, USA.
4. Egodage R.J.S., Hoover R., Waduge R., Liu Q. (2016). Impact of annealing on the hierarchical structure and physicochemical properties of waxy starches of different botanical origins. *13th International Hydrocolloids Conference,* Guelph, ON, Canada.

**Awards**

2018 Third place, Student poster competition. *The International Society for Nutraceuticals and Functional Foods (ISNFF),* Vancouver, BC, Canada.

2018 Third place, International Division's Melcolm Bourne Student Poster Competition. *IFT18 Annual Meeting & Food Expo,* Chicago, IL, USA.