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Effects of Chemical and Enzymatic Modifications on the

Starch-Inclusion Complex Formation

Effects of Chemical and Enzymatic Modifications on the
Starch-Inclusion Complex Formation

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Food Science

by

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ABSTRACT

Starch-inclusion complexes have been proposed as delivery tools for bioactive molecules; however complexation yield is generally low with low solubility, which may limit the bioavailability of the included molecule. It was proposed that chemical (acetylation) and/or enzymatic (isoamylase and β-amylase) modifications of starches prior to complex formation with fatty acids of different structures, including stearic, oleic and linoleic acid, may help increase complexation yield and solubility of the resulting starch complexes. Potato starch had a significantly higher complexation yield than common corn and high amylose (~70%) corn starches after debranching combined with a β-amylase treatment. Debranched waxy maize starch and potato amylose displayed exothermic co-operative binding with hexanoic acid during the isothermal titration calorimetry (ITC) measurements. Acetylation improved the solubility of starch complexes and increased the amount of included fatty acids in both soluble and insoluble starch complexes compared with the unacetylated starches. The degree of acetylation was generally higher for the soluble complexes than for the insoluble ones, which also increased recovery of soluble complexes but decreased the recovery for the insoluble complexes. Complexation between acetylated starches and fatty acids decreased with an increase in degree of unsaturation following the order of stearic acid > oleic acid > linoleic acid. Acetylation of debranched starch alone or in combination with the β-amylase treatment can be employed to increase complexation yield as well as to improve the solubility of complexes. This may potentially prove beneficial in food or pharmaceutical application because an increase in solubility can improve complex digestibility, and this may ultimately increase the bioavailability of the included molecules.

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LIST OF PUBLISHED/PUBLISHABLE PAPERS

I. Arijaje, E. O.; Wang, Y.-J. Effects of Botanical Source and Enzymatic Modifications on the Starch-Stearic Acid Complex Formation.- To be submitted - Chapter 1

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III. Arijaje, E. O.; Wang, Y.-J.; Shinn, S.; Shah, S.; Proctor, A. Effects of chemical and enzymatic modifications on starch-stearic acid complex formation. *J. Agric. Food Chem.* **2014**, *62*, 2963−2972 - Chapter 3. Reprinted with permission from American Chemical Society, See page 125.

IV. Arijaje, E. O.; Wang, Y.-J. Effects of Chemical and Enzymatic Modifications on Starch-Oleic Acid Complex Formation - To be submitted - Chapter 4

V. Arijaje, E. O.; Wang, Y.-J. Effects of Chemical and Enzymatic Modifications on Starch-Linoleic Acid Complex Formation - To be submitted - Chapter 5

I. GENERAL INTRODUCTION

Starch is the primary source of carbohydrate and energy in plants, and the major component of most foods.[1,2] Starch is a homoglucan and consists of two main components, i.e. amylose of an essentially linear molecule with few branches, and amylopectin of a highly branched structure. Irrespective of the sources, starch consists of a similar backbone of repeating α-D-glucose units linked by α-D-(1→4) glucosidic linkages with branching points linked by α-D-(1→6) linkages. In dilute solution, amylose assumes a helical conformation with a hydrophilic exterior and a hydrophobic interior, which enables its interactions with hydrophobic compounds to form amylose-inclusion complexes.

Bioactive compounds are extranutritional constituents that are found in food of both plant and animal sources and present in small quantities,[3] such as flavonoids, phenolics, and anthocyanins. Numerous studies have shown the health benefits of these bioactive compounds, such as control of insulin sensitivity, positive effects on cardiovascular disease factors, reduction of atherosclerosis, treatment of diabetes, and antioxidant and anti-cancer properties.[4,5] However most bioactive compounds are unstable and easily degraded under thermal or oxidative stress, therefore studies have been conducted to improve stability of these bioactive compounds by including them in an inclusion host.

Inclusion complexes of bioactive compounds have shown to be a viable means of protecting the bioactive compounds from oxidation and degradation with starch and cyclodextrins being the most studied inclusion hosts. Cyclodextrins (CDs) are cyclic oligosaccharides composed of 6, 7 or 8 glucose units to yield α, β and γ-cyclodextrin, respectively. CDs have a truncated cone shape with the outer part being hydrophilic while the inner part being hydrophobic. This arrangement enables CDs to form inclusion complexes with a

wide range of hydrophobic molecules. Among CDs, β-cyclodextrin is the most accessible and widely used mainly because of its cost. The inclusion complexes with CDs usually have poor solubility, which results in reduced bioavailability. Chemical modification of CD helps to reduce its limitations but it also drives up costs of production. Starch therefore offers a better alternative as an inclusion host, because of its abundance in nature, ease of digestibility and low costs of modifications.

Despite numerous studies on the formation of amylose inclusion complexes over the years, only more recently has inclusion complexes of amylose and bioactive compounds been considered as a delivery system.[6,7] Amylose has been shown to forms inclusion complexes with some bioactive compounds such as salicylic acid and analogues,[8] p-aminobenzoic acid,[9] ibuprofen and warfarin,[10] ascorbyl palmitate, retinyl palmitate, phytosterol esters[11] and conjugated linoleic acid.[6,12] Although these studies have demonstrated the inclusion complexation of starch with bioactive compounds, the yields of complexes reported are very low. In addition, upon the complex forming, the starch complex becomes insoluble and precipitates out of solution. This subsequent precipitation of complex limits the solubility, digestibility and subsequently may limit the bioavailability of the included molecule.

The goal of this study was to improve the starch-inclusion complexation yield and the solubility of the resulting complex by determining the preferred molecular structures and modifications that would enhance and stabilize the formation of starch-fatty acid complexes and consequently help to improve its solubility. Fatty acids with different degrees of unsaturation were used as model compounds. The specific research objectives of this study were to:

1. Determine the effect of enzymatic modification (isoamylase without or with a β-amylase treatment) of starch from different botanical sources on starch-stearic acid complex

formation and to determine the effects of molecular size on the formation and solubility of starch-stearic acid complexes,

2. Investigate the complexation of starch chains from different botanical sources with fatty acid (hexanoic acid) using isothermal calorimetric titration, and
3. Determine the effects of a combination of chemical (acetylation) and enzymatic modifications (isoamylase without or with a β-amylase treatment) of starch on complexation yield and properties of both soluble and insoluble complexes with fatty acids of different chemical structures, including stearic (18:0), oleic (18:1) and linoleic (18:2) acids.

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II. LITERATURE REVIEW

Starch

Starch serves as a main energy source in human diets and plays an integral part in various foods because of its availability and diverse physicochemical and functional properties.[1] Starch occurs naturally as granules and is insoluble in water because of its semi-crystalline structure. Commercial starches are usually extracted from corn, potato, tapioca, wheat, and rice. The appearance and characteristics of starch from different plant sources vary greatly and are summarized in Table 1. Most starches are composed mainly of a mixture of two polymers: amylose, a mixture of essentially linear polysaccharides with few branches, and amylopectin, a mixture of highly branched polysaccharides. Both components are homopolymers of α-D-glucopyranose with the linear chains linked by α-D-(1→4) glucosidic linkages and the branch point linked by α-D-(1→6) linkages. Non-carbohydrate components, such as lipids, proteins and phosphorus, are present in minute amounts[2,3] but have strong impacts on the properties of the starch.

The functionality of starch is principally affected by the ratio of amylose and amylopectin due to their distinct structures and properties. The ratio of amylose and amylopectin from different sources differ[4,5] (Table 1) and may slightly also depend upon the methods of extraction or measurement. Native starch granules exhibit three distinct wide angle powder X-ray diffraction patterns, including the A-, B- and C-type (Figure 1).[6] Cereal starches such as maize and rice exhibit the A-type polymorph; tuber starches like potato display the B-type; the C-type is found in bean or root starches.[7]

Table 1. Characteristics of starch from different botanical sources[8,9]

Starch	Type	Granule Shape	Granule Size (μm)	X-ray Pattern	Amylose (%)	Amylopectin (%)
Barley	Cereal	Lenticular (A-type) Spherical (B-type)	15-25 2-5	A	~28	~72
Maize (waxy)	Cereal	Spherical	2-30	A	~0	~100
Maize (normal)	Cereal	Polyhedral	2-30	A	~27	~73
Maize (high amylose)	Cereal	Irregular	2-30	B	50-75	25-50
Pea	Legume	Oval/Compound	5-50	C	~25	~75
Potato	Tuber	Lenticular	5-100	B	~21	~79
Rice	Cereal	Polyhedral	3-8	A	0-30	70-100
Tapioca	Root	Spherical/lenticular	5-45	A	~17	~83
Sago	Cereal	Oval	15-35	A	~25	~75
Wheat	Cereal	Lenticular (A-type) Spherical (B-type)	>10 2-10	A	~28	~72

The V-type polymorph is another crystalline structure that is composed of a single amylose helix,[10] and is formed primarily during recrystallization or complexation of amylose. The A and B-type polymorphs differ in the packing density of their double helices, the geometry of their single cells units, and the amount of water that is bound to the crystal structure. The A-type polymorphic starch possesses a monoclinic unit cell, the B-type has hexagonal unit cells, and the C-type is a mixture of the A- and B-type[11,12] (Figure 2).

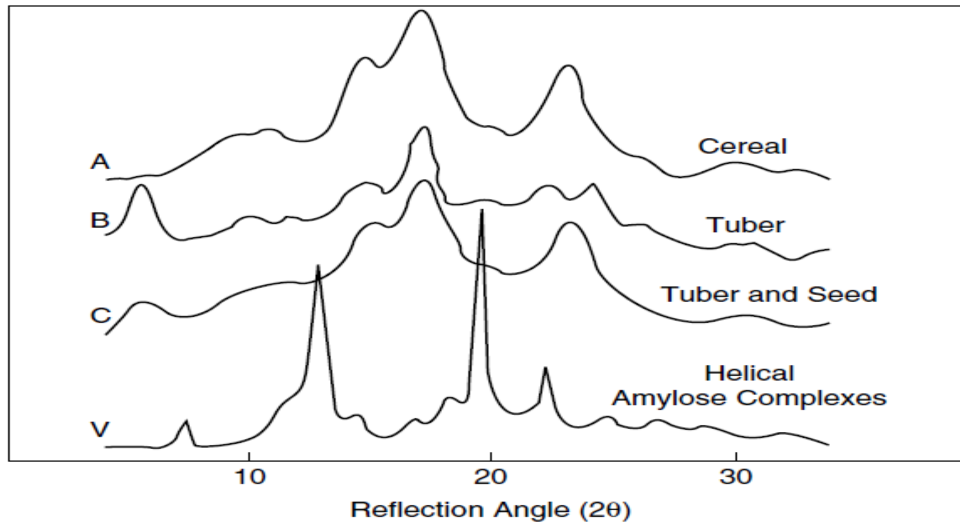

Figure 1. Wide angle X-ray diffraction patterns of the A-, B-, C and V-type starches and their sources.[13]

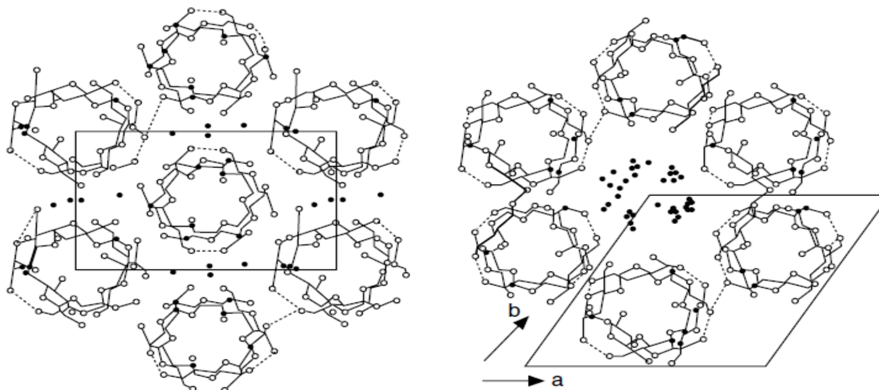

Figure 2. The helix packing in the A-type (left) and the B-type (right) starch. Water molecules are indicated by dot and H-bonds by dashed lines.[11]

The A-type polymorph is more densely packed and possesses less water with 8 water molecules per unit cell, while the B-type has 36 water molecules per unit cell.[8,14] The average chain length of the amylopectin in the A-type starches consists of 23-29 glucoses, which is shorter than that of the B-type starches of 30-44 glucoses,[15] whereas that of the C-type starch of

26-30 glucoses falls between the A- and B-types.[16] The A-type starch has a higher melting temperature and thus is more stable when compared with the B-type starch.[17]

Native starch is semi-crystalline in nature, and it is generally accepted that amylopectin is responsible for its crystallinity. The linear short chains in amylopectin intertwine to form double helices, which tend to form clusters that pack together to line up perpendicular to the growth rings. The growth rings originate from the hilum to the periphery of the granule in a radial arrangement.[1,18,19] The arrangement of the cluster packing forms the alternating crystalline and amorphous lamella (Figure 3).[11,20] The crystalline lamella corresponds to the double helices, and the amorphous lamella corresponds to the branching regions that consist of amylose and amylopectin branching points.[1]

The possible arrangements of amylose and amylopectin in the granule have been proposed. Montgomery et al.[21] proposed that a large amount of amylose existed in the amorphous region with a small fraction residing in the amorphous lamella. Later, Nikuni[19] proposed another model where amylose existed freely without interaction with amylopectin within the amorphous and semi-crystalline regions. More recent studies have shown that amylose co-crystallized with amylopectin and therefore amylose also resided in the crystalline lamella.[22,23] As a result of the co-crystallization, amylose might disrupt the packing of amylopectin by pulling two adjacent amylopectin chains closer, therefore an increase in amylose content resulted in a decrease in the amorphous lamella.

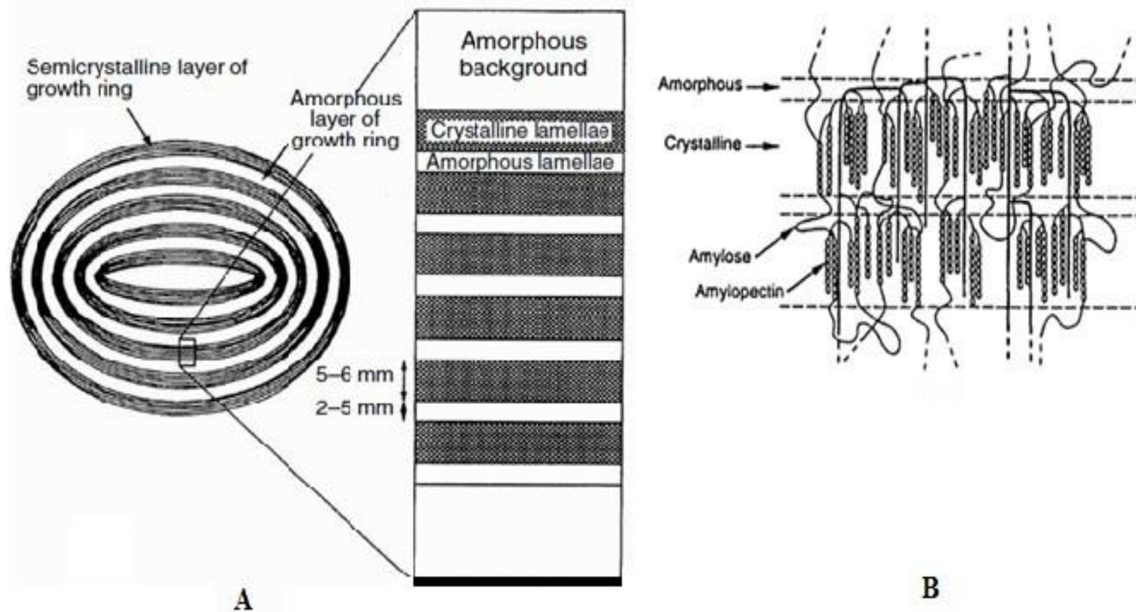

Figure 3. Expanded view of internal structure showing concentric rings of alternating amorphous and crystalline lamella (A), and the location of amylose and amylopectin in a starch granule (B).[20,24]

Amylose

Amylose is an essentially linear molecule made of glucose units linked together by α-D-(1→4) glucosidic linkages(Figure 4) with about 0.3-0.5% branched fractions linked by α-D-(1→6) linkages. The presence of α-D-(1→6) linkages in amylose has been confirmed because of the presence of beta-limit dextrins after β-amylase hydrolysis of amylose from potato and cereal sources,[25] but the amount of branches present varies with the plant source.[26]

There are abundant hydroxyl groups present in the amylose molecule, which make it hydrophilic. However, at the same time because of its linear structure, mobility and numerous hydroxyl groups, amylose is able to interact with adjacent amylose chains by forming hydrogen bonds. This reduces the affinity of amylose for water and enhances the association between

amylose molecules.[27] This amylose-amylose interaction is known as retrogradation, which has great impacts on starch properties.

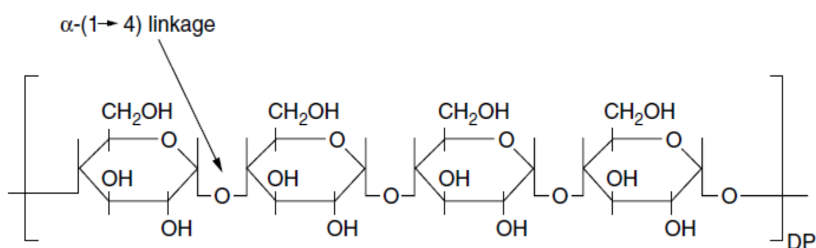

Figure 4. The glucopyranose units and α-D-(1→4) glucosidic linkages in amylose.[27]

The molecular size of amylose in degree of polymerization (DP) ranges between 200 and 20,000 glucose units and differs with starch source (Table 2). The average molecular weight of amylose ranges from 30,000 to 3,200,000 g/mole,[28] and amyloses from cereal sources generally have a lower molecular weight than those found in tubers.

Amylose can exhibit a helical configuration and in dilute solutions forms inclusion complexes with suitable organic guest molecules such as iodine.[29] The amylose helix can consist of 6, 7 or 8 glucose units per turn depending on the size of the guest molecules.[30-33] The internal diameter of the helix is approximately 0.5 to 0.85 nm in pitch height,[31,33] and the left-handed helix forms a hydrophobic cavity that encourages the formation of inclusion complexes with hydrophobic molecules.

Table 2. Amylose molecular size from different starch sources.[34]

Starch Source	Degree of Polymerization (DP)
Rice (indica)	3420
Maize	2500
High amylose corn (70% amylose)	1990
Wheat	3480
Barley	4470
Sago	4380
Tapioca	6680
Potato	6360

Amylopectin

Amylopectin is the predominant component ranging from 70-80% in most starches. Amylopectin molecules are highly branched and consist of linear glucose chains linked by α-D-(1→4) linkages with branching points connected by α–D-(1→6) linkages (Figure 5). Amylopectin is among one of the largest biopolymers in nature ranging from DP 300,000 to 3,000,000 glucoses with a molecular weight of about 10^6 - 10^9 g per molecule.[35] The type of crystalline structure displayed by the starch is governed by the chain length of the branches present in the amylopectin molecule[15] as previously discussed. Amylopectin molecules with longer average chains crystallize into the B-type starch while those with shorter average chains yield the A-type starch.

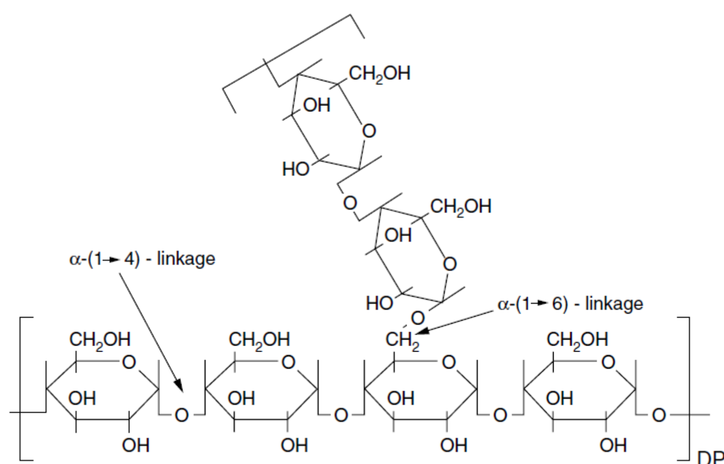

Figure 5. Structure of glucopyranose units and both α-D-(1→4) and α-D-(1→6) glucosidic linkages in amylopectin.[27]

Various models have been proposed to describe the amylopectin structure over the years. The cluster model proposed by French[18] (Figure 6a) has been accepted as the most probable one. The model suggests that the exterior chains of the amylopectin exist as double helices, which contribute to the crystalline lamellae that are separated by the amorphous lamellae. The model also suggests that an increase in the number of clusters increases the molecular weight of the amylopectin. Amylopectin is made up of three main chains: A, B and C chains.[36] The shortest, outermost chains are the A chains, and they carry no other chains; the B chains carry A chains or other B chains or bind to C chains; the C chains contain the sole reducing terminal residue in each amylopectin molecule, and carry only B chains. The cluster model was further modified by Hizukuri[37] (Figure 6b) based on the HPLC results of debranched starch by dividing B chains into B1-4 fractions with B1 chains of DP 20-24, B2 chains of DP 42-48, B3 chains of DP 69-75, and B4 chains of DP>100. A single cluster is made of A and B1 chains, B2 chains extend into two clusters, B3 chains are across three clusters, and B4 chains are across four clusters.[37]

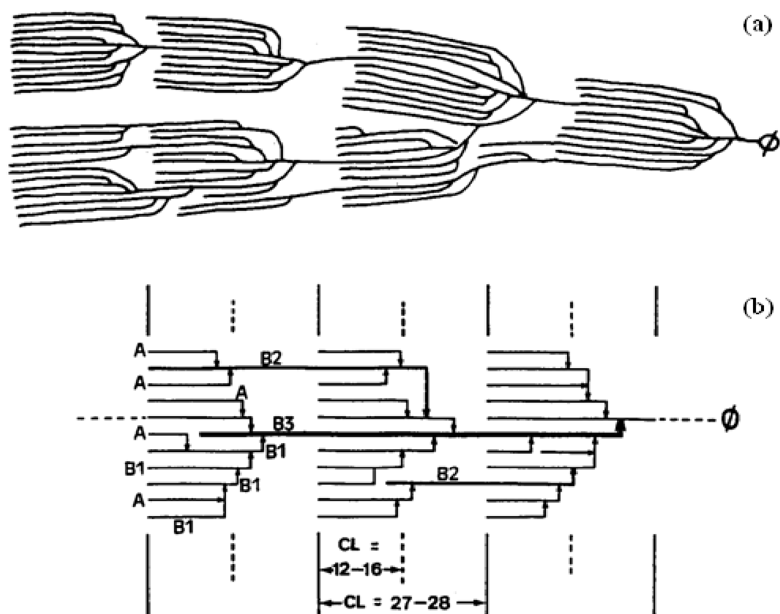

Figure 6. The cluster model of amylopectin structure adapted from French[18] (a) and Hizukuri[37] (b).

Amylose-Inclusion Complex

The formation of inclusion complex between amylose and iodine was first reported by Colin & de Claubry.[29] Amylose forms a helical structure with a cavity that can include iodine atoms to give the blue color. On forming the helix structure, the inner cavity becomes highly hydrophobic due to the arrangement of the polar portion of amylose been aligned outwards (hydrophilic) and the less polar portion been oriented inwards to form the hydrophobic cavity that favors hydrophobic interaction.[10] The interaction between amylose and iodine has been proposed as a method to determine the amylose content due to a characteristic color that is formed on complexation.[10,38-40] The intensity of the color formed by the amylose-iodine complex has been found to vary with amylose chain lengths.[38] Bourne et al.[41]proposed a method referred to as the "Blue Value" for measuring the amylose content by measuring the absorbance of the blue color formed by the complex at a wavelength of 680 nm. These same amylose-iodine

interactions have also been exploited in potentiometric iodine titration, which is a method often employed to determine the amylose content.[42]

Bailey and Whelan[38] determined that the relative chain length of amylose could also be determined by the amylose-iodine reaction as the color and λ_{max} of the complexes change depending on amylose chain length and the helix cavity. The λ_{max} of the complex increases with increasing amylose DP. Amylose with DP<12 had no color; DP12 was the threshold value for forming amylose-iodine red color; DP 30 stained red purple with λ_{max} = 550 nm; DP >52 had a blue green color with λ_{max} = 595nm. This color intensity remained at λ_{max}= 645 nm even at a higher DP of 568. Banks et al.[43] reported the estimated λ_{max} limit for starch-iodine complex was 642 nm, while John et al.[44] estimated it at 650 nm.

Amylose also forms complexes with other compounds such as some alcohols such as 1-butanol, lipids, flavors, DMSO, surfactants and salicylic acid.[45-49] The interaction between amylose and alcohol has been the basis of separating amylose from amylopectin by selective precipitation of amylose using butanol.[45]. Schoch[42] studied amylose inclusion complexes and reported that the complexation between amylose and a compound was reversible. Banks et al.[25] reported that the resulting complex was crystalline in nature and possessed a V-type X-ray diffraction pattern. The study by Rondeau-Mouro et al.[49]reported that guest molecules were not only included in the helix cavity of amylose (intra-helical) during complexation, but also trapped between the amylose helices, which contributed to the inter-helical complex formation.

Amylose-Lipid Complex

The amylose-lipid complex (Figure 7) was first reported by Schoch and Williams.[50] They reported that the molecular structure formed between amylose and fatty acids was a molecular

complex similar to that of amylose with iodine and alcohol, and the interaction was not due to surface adsorption. The complex usually forms during heating of starch in the presence of added or naturally present lipid[51]and after complexation, the complexes separate out and can be recovered by centrifugation.[50]

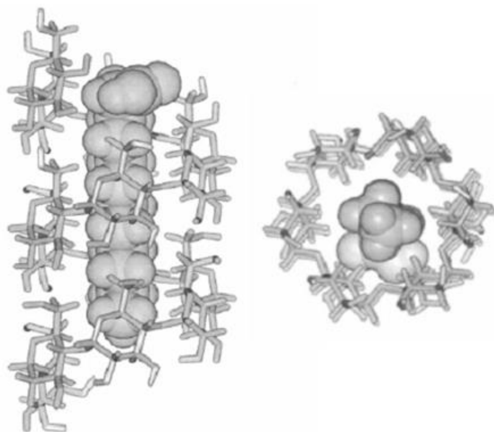

Figure 7. Structure of Amylose-lipid complex.[8]

Mikus et al.[46] found that only amylose with a helical structure would bind fatty acid, while the extended form would not form a complex irrespective of the size of the amylose or fatty acid. The conformation of amylose, whether extended or helical, is of great importance for complexation, and is dependent on factors such as pH and temperature of the solution where complexation occurs. Amylose exists as a helix in acidic or neutral conditions but displays a fully extended coil when the pH approaches 12 because hydrogen bonds are weakened with increasing pH and temperature.[52-54]

The hydrophobic interactions between amylose and fatty acid have been known to increase the hydrocarbon chain length of fatty acid increases.[55] The complexation between amylose and fatty acid is dependent on many factors such as amylose chain length, lipid structure, and reaction conditions (e.g. temperature, pH, and solvent), and fatty acid solubility.[31,50,56-58] Evans[59] reported that the temperature at which the complexation reaction was

carried out determined if amylose would form inclusion complex with itself or with the guest molecule. Amylose inclusion complexation seemed to be favored over amylose-amylose interaction at high temperatures, which was attributed to an increase in amylose mobility and ability to interact with the guest molecules (lipids) rather than with other amyloses.

Godet et al.[57] reported that for amylose a minimum of DP 20-30 glucose units is required to complex with caprylic (C8:0) and lauric (C12:0) acid and about 30-40 glucose units to complex with palmitic acid (C:16:0). These chain lengths were reported to be sufficient to accommodate two fatty acids at a time. Fatty acids and mono- and diacyl glycerols can form complexes with amylose, while the triacyl glycerols cannot.[60] The stability of complexes formed decreased with increasing unsaturation of the fatty acids.[47,61] Tufvesson et al.[62] (2003b) reported that complexes formed by fatty acids were thermally more stable than those formed by monacyl glycerols. Longer fatty acids (C≥12) showed improved complexation with amylose, which was due to the fact that longer fatty acids are less soluble in the complexing solution and tend to interact more with the hydrophobic cavity of the amylose. They also found that anionic fatty acids favored complex formation of the longer fatty acids (≥C:12) when compared with the uncharged ones.

Amylose-lipid complex can exist as Type I or Type II.[51,56] The Type I complex has a lower melting temperature (T_m) of around 90 °C and the Type II with a T_m of around 110 °C. The Type I complex is known to form at lower temperatures of about 60 °C, and therefore is less ordered and low in heat stability.[33,63] The Type I complex is formed when rapid nucleation of the amylose-lipid complex occurs at around 60 °C,[56] whereas the Type II is formed with continuous heat treatment and re-arrangement of the complexes at higher temperatures of about 90 °C[61] (Figure 8). The Type II complex is more heat stable because the nucleation rate is low, and this

allows for sufficient propagation.[63] Tufvesson et al.[64] reported that a prolonged heat treatment was required for the formation of Type II complexes with long monoglycerides such as glycerol monopalmitin (GMP) and glycerol monostearin (GMS), but was not required for those with shorter chain such as glycerol monocaprin (GMC), glycerol monolaurin (GML), and glycerol monomyristin (GMM).

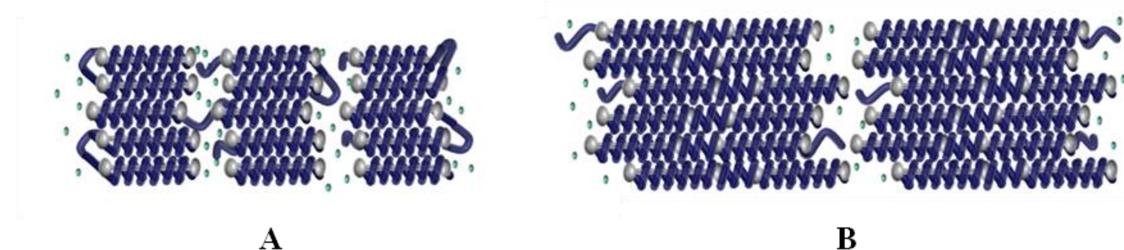

Figure 8. Type I (A) and Type II_a (B) amylose-lipid complex.[65]

The Type II complex has been further classified into two groups: Type II_a and II_b (Biliaderis et al, 1986). The Type II_a is formed at a temperature ≥ 90 °C and has a T_m of 114-121 °C, whereas the Type II_b is formed after further annealing of the Type II_a at 105-115 °C to become a more thermostable complex than the Type II_a with a T_m of 121-125 °C.[61] Biliaderis and Galloway[56] described the conversion of Type II_a into Type II_b as a classical annealing process which occurred first with the partial melting of crystallites followed by recrystallization.

Although studies have shown that the complexation between amylose and a compound is reversible,[42] there are contradictory results on the digestibility of amylose-lipid complex. Holm et al.[66] showed that the amylose-lipid complex was hydrolyzed by the hog pancreatic α-amylase and absorbed in the gastrointestinal tract, however the rate of hydrolysis was slower compared with the uncomplexed amylose. Gelders et al.[33] prepared complexes with amylose of varying degrees of polymerization (DP 60, 400, and 950) and lipids (docosanoic acid (C22:0) and glyceryl monostearate) in dimethyl sulfoxide (DMSO)-water solvent at 60°C or 90°C for 4 hr. The

complexes were subjected to porcine pancreatic α-amylase and there was a decrease in the enzymic hydrolysis with increasing amylose DP or complexation temperature. The hydrolysis by α-amylase or acid follows the order of Type I > Type II_a > Type II_b with their temperature stability in the opposite order.[33,67] The rate of hydrolysis was also influenced by both lipid chain length and degree of saturation in which resistance increased with increasing lipid chain length and decreased with increasing unsaturation.[33,47] The complexes formed between amylose and lipid offers various benefits such as preventing the lipid from oxidation through stabilization. Therefore it has been proposed that the amylose-lipid complex can serve as a form of controlled or targeted release mechanism for lipids.[53,68,69] Szejtli and Banky-Elod[53] complexed amylose and a mixture of both saturated (palmitic and stearic acid) and unsaturated fatty acids (linoleic, linolenic, and oleic) and reported that the complexed unsaturated fatty acids were completely protected from oxidation even in the presence of oxygen. Yang et al.[69] reported that the oxidation of complexes of amylose and conjugated linoleic acid (CLA) was reduced, and complexes were fully released over a 15-hr period under simulated stomach and small intestine conditions.

Despite the numerous studies, the conditions required for a high yield of amylose-complex formation are yet to be fully elucidated. Most studies have reported low yield of complexed lipids usually ranging from 1-5% of total complex weight.[53,61,70]

Bioactive Compounds

Bioactive compounds are important extranutritional constituents due to the various benefits they confer on human health such as anti-oxidative and anti-cancer properties. Many are present in food mostly in small quantities and are very sensitive to thermal and oxidative stress, resulting in rapid degradation during processing or storage.[58] Some natural bioactive compounds such as polyunsaturated fatty acids (PUFAs), phenolics, flavonoids, anthocyanins are still been

studied to reduce the rate of their degradation. For example, PUFAs can easily be oxidized in the presence of atmospheric conditions even with little or no processing. As a result of the numerous health benefits, it has become imperative to research ways of effectively stabilizing and delivering these components.

Recently, CLA has become an actively studied bioactive compound. CLA was accidently discovered by Pariza and his co-workers. Pariza et al.[71] first reported on the health benefits of CLA although the identity of CLA was still unknown, it was the group led by Ha et al.[72] that later identified and discovered the structure of the CLA isomers. CLA exists as a mixture of positional and geometric isomers of linoleic acid (C18:2), and has two double bonds separated by a single bond. Among the isomers, only a few are biologically active such as *9c, 11t*-CLA, *10t, 12c*-CLA, *9t, 11c*-CLA, which are naturally found in beef, lamb and dairy products. Over the years, the health benefits of CLA has been reported in areas such as modulation of plasma lipids, improvement of plasma cholesterol status, anticarcinogenic properties, reduction of colorectal cancer, and control of obesity and diabetes.[72-74] Recently, Jain and Proctor[75] synthesized *trans-trans* CLA isomers by photoisomerization of soy oil and studied its nutritional effects on zucker rats.[76] They reported that the *trans-trans* CLA-rich soy oil reduced serum cholesterol and low-density lipoprotein-cholesterol levels by 41 and 50%, respectively, when compared to the obese rats fed with a control diet containing linoleic acid. However, similar to other unsaturated fatty acids, t*rans-trans* CLA is oxidatively unstable, therefore studies are need to stabilize these isomers in order to realize their health benefits.

Cyclodextrins

Cyclodextrin (CD), first described by Villers,[77] is a cyclic oligosaccharide consisting of 6 (α-cyclodextrin), 7 (β-cyclodextrin) or 8 (γ-cyclodextrin) glucopyranose units connected by α-D-

(1→4) linkages, and the number of glucose units present in the CD determines the dimension and size of the cone cavity (Figure 9). Cyclodextrins have the shape of a truncated cone with the wider end formed by the secondary 2- and 3-hydroxyl groups and the narrow end formed by the primary 6-hydroxyl group.[78] The cavity of the cone is lined with hydrogen atoms and glucosidic oxygen bridges, which have non-bonding electron pairs directed toward the inner cavity.[79] This results in a high electron density in the cavity, which is responsible for the relatively hydrophilic exterior and the lipophilic interior of CDs. Because of these unique properties, CDs favor the formation of inclusion complexes.[79-81] However natural cyclodextrins, in particular β-CD, have been reported to exhibit limited aqueous solubility of 12.8, 1.8, and 25.6 grams per 100 mL of water for α, β, and γ-CD, respectively.[82] The much reduced solubility of β-CD was reported to be due to its stronger intermolecular hydrogen bonding that diminishes its ability to form hydrogen bonds with surrounding water molecules.[80,83]

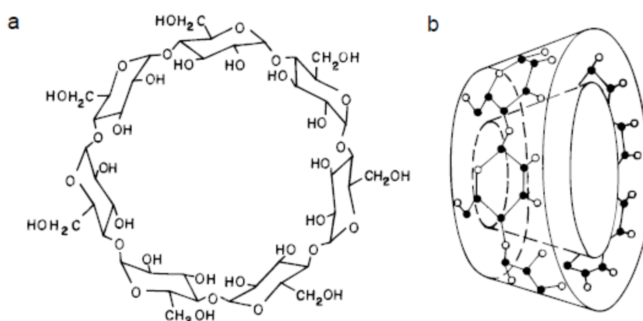

Figure 9. Chemical structure (a) and toroidal shape (b) of β-cyclodextrin.[80]

Modifications have been carried out to yield various derivatives such as 2-hydroxypropyl-β-CD, methylated β-CD, branched β-CD such as glucosyl β-CD and sulfobutylether β-CD sodium salts to improve the properties of CDs such as solubility (Loftsson et al, 2005). After complexation with modified CDs, the physiochemical properties of guest molecules such as solubility, stability of labile guests against degradation, visible or UV light

sensitivity, chromatographic separations, taste and controlled release of drugs and flavors become significantly altered and improved.[85] Although these modifications help overcome a variety of limitations, they also drive up cost of commercialization of CD complexes.

Several hypotheses have been proposed for the driving forces behind complexation of CDs with other compounds. During complexation with CD, the guest molecule is included in the cavity with the release of enthalpy-rich water molecules from the cavity as the driving force for the complexation reaction.[85] Other driving forces that have been suggested include van der waals forces, hydrogen bonding between polar groups of guest molecule, hydrophobic interactions between hydrophobic portion of guest molecule and CD cavity, release of the ring strain in CD molecule, and changes in solvent-surface tension.[86-89] Loftsson and Brewster[80] proposed that a combination of these reactions instead of one simple construct might be needed to drive the complex formation.

Inclusion Complexes of Cyclodextrins and Bioactive Compounds

Native or derivatized CDs have been used widely in the pharmaceutical, food, cosmetic and other industries.[90] Bioactive compounds that have been complexed with CDs are indomethacin (an anti-inflammatory drug),[91] nalidixic acid (an antibacterial drug),[92] iriquinone (an anti-cancer drug), rutin (a flavonoid),[93] β-lapachone (a natural anticancer drug)[94] ferrocene (an antiknocking agent in petrol engine), nicotine, sodium dicolofenac (eye drops used to reduce redness and swelling after cataracts treatment)[95] and aromas (febreze®). However, the effectiveness of CD as a complexing agent is limited mainly by factors such as cost and restrictiveness of the cavity. Therefore it is important to explore alternatives to CDs with a lower cost yet improved binding.

Inclusion Complexes of Amylose and Bioactive compounds

Despite numerous studies of the formation of amylose inclusion complexes over the years, only more recently has inclusion complexes of amylose and bioactive compounds been considered as a delivery system.[70,96] Inclusion complexes of amylose and salicylic acid have been reported by Oguchi et al.[48] They found that amylose (M_w= 1,310,000) complexed one molecule of salicylic acid per helical turn when 10% salicylic acid was used in complexation and displayed a 7_1 helix structure (amylose helix with seven glucose units per turn). However when the quantity of salicylic acid used in complexing was increased to 30%, two molecules were complexed per amylose helical turn displaying the 8_1 helix structure (amylose helix with eight glucose units per turn). The complexes formed with the 7_1 helix complex were more stable because the salicylic molecules were more tightly bound than the ones found in the 8_1 helix complex.

Lalush et al.[70] investigated complexes formed from amylose and conjugated linoleic acid (CLA) using two different solvents of dimethyl sulfoxide (DMSO)/water and KOH/HCl at three different complexation temperatures of 30, 60 and 90 °C. Both amylose and CLA were soluble in DMSO/water, which encouraged complex formation. On the other hand, in the KOH/HCl solution, KOH helped to foster the solubility of CLA through ionization while HCl was added to neutralize the reaction. However, complex formation was much reduced in the KOH/HCl solution since the solubility/dispersability of CLA was less in water than in the organic solvent, therefore there was limited amount of CLA available for complex formation. Complexes formed using DMSO/water provided greater oxidative stability and exhibited higher weight yield compared to those formed in the KOH/HCl solution, with complexation temperature of 30 or 90°C producing the highest percentage of CLA per gram of complex of 3.8% and 3.3%,

respectively. Within the DMSO/water treatments, the reaction temperature of 60 °C resulted in the least amount of CLA per gram of complex (2.6%) with the least stability. Within the KOH/HCl treatment the complexes formed at 60 °C also contained the least amount of CLA per gram of complex (1.9%), but was the most stable. All complexes were tested for susceptibility to enzymic hydrolysis under simulated stomach conditions, and the degree of hydrolysis of all complexes followed the order of pancreatin (~100%) > α-amylase (~87%) > amyloglucosidase (~37%) > β-amylase (~8.5%).

Yang et al.[69] compared complexes formed from amylose or β-CD with CLA and reported that the yield and complexation percentage of amylose-CLA was 71.9% and 1.4%, respectively, while those of β-CD-CLA was 42.3% and 7.7%, respectively. The amylose-CLA complex showed a better stability against oxidation than the BCD-CLA complex when peroxide values were compared for the two complexes. Under simulated stomach and small intestine conditions for 15 h, the enzymic hydrolysis and release percentage were 87.50 and 95.61% for the amylose-CLA complex and only 27.92 and 15.96% for the BCD-CLA complex. This implies that amylose was more effective in the protection of guest molecules and release of CLA from the complex.

Recently, Lay Ma et al (2011) examined the inclusion complexes prepared from amylose, amylopectin and high amylose maize starch with three bioactive components (ascorbyl palmitate, retinyl palmitate, and phytosterol esters). They found that amylopectin only complexed with retinyl palmitate, while amylose and high amylose maize starch complexed with all three fatty acid esters. They suggested that adsorption of some of the guest molecules with amylopectin could not be ruled out and that complexation of the guest molecules could only have occurred with the outer chains of amylopectin. In addition, amylose complexes displayed the V-type X-ray diffraction pattern, which was in agreement with previous studies (Karkalas & Raphaelides,

1986; Biliaderis & Galloway, 1989). The yield of complexation followed the order of ascorbyl palmitate > retinyl palmitate > phytosterol esters. The high yield for the ascorbyl palmitate-complex was attributed to the high solubility of ascorbyl palmitate mainly due to the presence of a hydrophilic portion (ascorbic acid). The solubility of guest molecule is an important factor on inclusion complex yield because the guest molecule has to be in solution to interact with amylose (Putseys et al 2010a).

Starch Modification

Native starch has limited applications in the industry because of its limited functionality, such as viscosity, texture, solubility, and stability towards pH, shear, temperature, and storage. To overcome these shortcomings, starch is often modified through physical, chemical and/or enzymatic means. Physical modification involves thermal treatments like annealing, spray drying, roll drying, and pregelatinization.[97] Chemical modification includes reactions such as conversion (acid-thinning, oxidation and dextrinization), crosslinking, and substitution (esterification and etherification). Enzymatic modification is achieved using amylases (α-, β-, or glucoamylase) and debranching enzymes (isoamylase and pullulanase) to hydrolyze starch.

Chemical Modification

Chemical modification alters the interactions between starch chains by modifying the hydroxyl groups on the anhydroglucose units (AGU) or the glucosidic linkages.[98] Chemical modifications may be combined according to the Code of Federal Regulations (CFR) to further improve starch properties. The properties of modified starch vary depending on the type and degree of modification. For substitution, there are three hydroxyl groups, C2, C3 and C6 (Figure 10) available for reaction in an AGU because the hydroxyl groups in C1 and C4 are involved in

glucosidic linkages. Therefore the maximum degree of substitution (DS), which is the average number of hydroxyl groups that are substituted per AGU, is three.

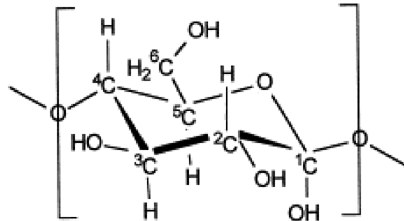

Figure 10. α-D-Anhydroglucose unit.

For substitution reactions such as hydroxypropylation where hydroxypropyl groups can react further with the reagent to form a polymeric substituent, molar substitution is used and could be greater than three.

Conversion

Conversion of starch weakens and degrades starch granules and thus results in reduced swelling and consequently viscosity in water.[97] During conversion, the primary attack occurs with hydrolysis at the α-D-(1→4) glucosidic linkages, but scission of some α-D-(1→6) bonds could also occur.[97] Conversion of starches is usually achieved by acid, hypochlorite, or a combination of acid and high temperature (pyroconversions or dextrinization).

Crosslinking

Crosslinks are formed by di- or poly-functional chemicals, which bridge between starch molecules and reinforce the hydrogen bonds responsible for granule integrity. Crosslinking agents allowed for food uses according to CFR include epichlorohydrin, phosphorus oxychloride, sodium metaphosphate and adipic/acetic anhydride. Most crosslinked starches have about one crosslink in every 1000 to 3000 AGU.[99] The level of crosslinking greatly influences

the starch properties, such as maintenance of granule integrity on swelling, increased film strength, resistance to shear and viscosity breakdown, and only low levels of crosslinking are required to maintain starch integrity.[97]

<u>Substitution</u>

Substitution involves stabilization of starch granule with the addition of ionic or non-ionic groups to prevent re-association of starch molecules and extending the shelf life of starch products (Knill & Kennedy, 2005). Most common types of substitutions approved by CFR are acetylation and hydroxypropylation for food applications.[102]

- Acetylation

Acetylation involves the esterification of acetic anhydride or vinyl acetate to starch in the presence of an alkali as a catalyst (Figure 11). The acetyl group is hydrophobic and yields a starch with decreased gelatinization temperature and increased swelling, solubility and storage stability. During acetylation it is important to maintain the pH at 8 - 8.4 when using acetic anhydride, and at pH 9 - 10 when vinyl acetate is used if optimum reaction efficiency is desired. The FDA regulation specifies a maximum of 2.5% acetyl content for acetylated starches used in food applications but the average reaction efficiency of acetylation is usually about 70% for granular starch.[103]

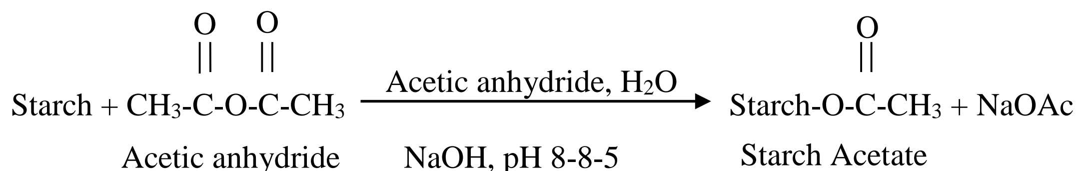

Figure 11. Acetylation of starch by acetic anhydride.

- *Hydroxypropylation*

Hydroxypropylation involves etherification of starch with propylene oxide (Figure 12) in the presence of an alkali as a catalyst and salt of 5-15% based on starch weight to prevent starch from swelling or gelatinization.[104] Hydroxypropylation takes approximately 24 h to complete and is about 60% efficiency with respect to the propylene oxide addition.

Hydroxypropyl groups are hydrophilic in nature and usually linked to the C-2 position of AGU.[104] The presence of hydroxypropyl groups weaken and disrupt the internal bonds responsible for granule integrity, thus altering the physiochemical properties of starch such as shelf life, freeze/thaw stability, cold water swelling and cold storage stability.[104] Shi and BeMiller[105] found that amylose was modified to a greater extent than the amylopectin, presumably because amylose was present in the amorphous regions. This was confirmed by Gray and BeMiller[106] who reported that the hydroxypropylation occurred first in the amorphous regions which are most accessible and then proceeded gradually to the crystalline regions.

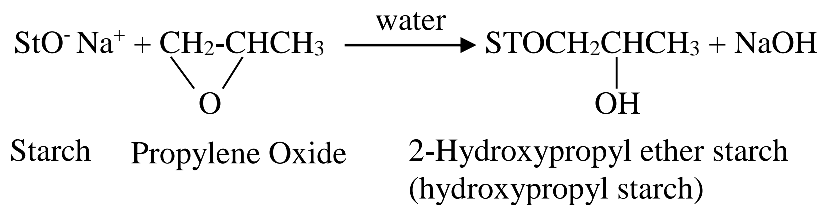

Figure 12. Substitution of starch by propylene oxide.

Hydroxypropylated starches are used in the food industry to improve freeze-thaw stability and also to yield products with low pasting temperatures.[104] Rutenberg and Solarek[98] reported that the effect of hydroxypropylation on gelatinization temperature was less when

compared to acetylation, but hydroxypropylation stabilized starch over a wide range of pH, and under low temperature conditions.

Enzymatic Modification

Enzymes are widely employed in the modification of starch to improve its properties, to study its fine structure, and to produce products such as corn syrups and sugars that can then be further converted to alcohol. Although enzymes can act on native starch, most times the modification is carried out using gelatinized starch because it is more susceptible to enzymes. Most commonly used enzymes are those allowed by the CFR and they include α- and β-amylase, glucoamylase, pullulanase, and isoamylase.

During starch hydrolysis by enzymes, the dextrose equivalent (DE) increases and the viscosity of the starch solution decreases. DE is an indication of the reducing sugar content present and is calculated as percent anhydrous dextrose of total dry substance.[5]The DE increases from 0 for starch and ultimately reaches 100 if all the starch is converted to dextrose. Maltodextrins are starch hydrolysates with DE < 20 while corn syrups have DE ≥ 20.

- α-Amylase

α-Amylase (1,4-α-D-glucan glucanohydrolase) is an endoglucosidase that attacks glucans internally away from the chain end. The action is random and results in a rapid drop in viscosity[107-111] and blue value.[34] It achieves this by hydrolysing mainly the α-D-(1→4) linkages, and its action is halted at branch point of α-D-(1→6) linkages of amylopectin or amylose as well as at α-D-(1→4) -linkages that are located very closely to the branch points.[5,34] However, α-amylase from *Thermoactinomyces vulgaris* has been reported to hydrolyse α-D (1→6) -linkages albeit weakly. α-Amylase can be extracted from many sources including bacteria, fungi, plants

and mammals, and its mode of action, properties and types of degradation products will depend on its source.[112] α-Amylase obtained from different sources exhibit different properties and the ones from *Bacillius amyloliquefaciens* and *Bacillius licheniformis* have been shown to exhibit high temperature stability. The products produced during the hydrolysis of amylose by α-amylase include glucose and maltose while varying α-limit dextrins, maltitriose or glucose are produced from the hydrolysis of amylopectin.[109,110] The relative concentrations of substrate and enzymes determine the type of degradation products produced.[109]

- β-Amylase

β-Amylase (1,4-α-D-glucan maltohydrolase) is an exoenzyme that occurs in many plants, such as barley, soy beans, and potatoes where it is often accompanied by the presence of α-amylases. It can also be produced from microbial sources. β-Amylase initiates hydrolysis by breaking the next to last glucosidic bond from the non-reducing end of a starch molecule to yield β-maltose until the reducing end is reached, or it experiences an α-D-(1→6) branch linkage.[5,108] The activity of β-amylase is therefore more pronounced on amylose than on amylopectin and its activity can be used to indicate the linearity of the glucose polymer or hydrolysate (Wurzburg, 1986).The complete breakdown of starch by β-amylase yields a mixture of maltose and β-limit dextrins.[34,53,99] The optimum conditions for β-amylase activity are pH 5.0-7.0 and temperature 45-70 ºC depending on source of the enzymes.

- Isoamylase

Isoamylase, an endoenzyme, is a debranching enzyme that has the ability to hydrolyze α-D-(1→6) linkages in starch to produce linear glucans.[114] The reaction is characterized by an increase in the iodine staining power and β-amylolysis limit.[115] Ueda and Nanri[116] reported that isoamylase from yeast (*E. intermedia)* had the ability to hydrolyze α-D-(1→6) glucosidic

linkages in both starch and glycogen completely but cannot hydrolyse α-D-(1→6) glucosidic linkages in pullulans completely.[117] In plants, reports have shown that isoamylases are also required for the normal synthesis of amylopectin, although the precise manner in which they influence starch synthesis is still unclear.[118]

- Pullulanase

Pullulanase works by exowise action to hydrolyze α-D-(1→6) glucosidic linkages of starch to produce maltotriose oligomers and finally maltotriose and trace amounts of maltotetraose. Pullulanase cleaves α-D-(1→6) glucosidic linkages between chains that contain a minimum of two glucose residues but not with a chain containing a single glucose residue, as with isoamylase.[119] However unlike isoamylase, it can hydrolyze pullulans completely but has limited hydrolytic power on glycogen.[117]

Modified Starches in Inclusion Complexation

Most modified starch used as inclusion hosts are often debranched to create more linear glucans for complexing with guest molecules. Yotsawimonwat et al.[120] investigated the precipitation reaction of debranched waxy rice starch complexed with lauric or stearic acid. Waxy rice starch was debranched with pullulanase, and the resulting debranched starch was complexed with varying amounts of lauric or stearic acid. They found that pH had a great effect on complex formation. At a pH that was below the pK_a of the fatty acids (~4.8), debranched starch precipitated in free form but at a pH above the pK_a, it precipitated as V_h-type complex, with the highest complexation at pH 7. Later, Yotsawimonwat et al.[121] examined the complexation between debranched waxy rice starch with varying short- and long-chain fatty acids (FAs). They reported that short chain FA with C8 was more soluble in aqueous solutions

and so readily complexed with debranched starch. However, the rate of complexation with the short chain FAs was reduced at pH ≥5 compared to FAs of longer chain (C10:0 – C18:0) at the same pH.

Recently, Hasjim et al.[122] prepared inclusion complex from isoamylase-debranched high amylose maize starch suspension (10%w/w) and palmitic acid at 95ºC for 1 h. They reported that the complexation yield increased when starch was treated with isoamylase prior to complexation, this was attributed to the presence of more linear chains produced by isoamylase. Zhang et al.[123] debranched high-amylose maize starch (70% amylose) with pullulanase for varying times (0, 2, 6, 8, 12 and 24 h), and the resultant debranched starches were complexed with lauric acid (10% w/w, dry starch base, dsb) in boiling water for 30 min. They reported that starch debranched for 24 h prior to complexation yielded the highest amount of complexed lipid, therefore they suggested that prolonged debranching could improve the formation of starch-lipid complexes.

Wulff and Kubik[124] investigated the chemical modification of amylose prior to complex formation. They determined that modification of amylose through hydroxypropylation improved the solubility of complexes and only slightly decreased the complexing ability of amylose. A degree of substitution of 0.075, which was equivalent to an average of one substituent for every 13 AGU, formed soluble inclusion complexes with fenchone. Kubik and Wulff[125] investigated the effect of crosslinking on amylose-inclusion complexation by using circular dichroism (c.d.) and isothermal microcalorimetry titration (ITC). Crosslinking stabilized the amylose complex and when amylose helix with six glucose units per turn was crosslinked, larger molecules that require a helix with 7 or 8 glucose units per turn could no longer be included in the helix. Wulff et al.[126] again prepared hydroxypropylated amylose (DS= 0.1) from amylose with varying DP to further investigate the practical applications for both soluble and insoluble amylose-flavor

complexes. The modified amylose was then complexed with flavor compounds, including 2-hexanone, linalool, fenchone and guaiacol. The use of hydroxypropylated amylose lead to an improved stabilization of flavor compounds by protecting them from oxygen and light. They suggested that the increased solubility of amylose in solution improved complexing efficiency, thus resulting in the improved stability of complexes. Inclusion complexes of the flavors were very stable over a one-year period. Nevertheless, low water activity was required for flavor stability because there was fast removal of flavors in the presence of water activity above 0.5.

Isothermal Microcalorimetry Titration

Isothermal titration calorimetry (ITC) (Figure 13) is a method that has been used to characterize the energetics of interaction on a molecular level[127] and this can also be done over a range of temperatures.[128] Titration calorimetry was first described by Hansen et al.[129] and Christensen et al.[130] However, it was not until 1979 that Langerman and Biltonen[131] published data on the use of microcalorimeters for biological chemistry. The calorimeter measures the heat output or uptake of a binding process, therefore, the heat measured can be used to express the extent of interaction occurring at equilibrum during titration.[128] ITC provides a rapid method for the accurate and direct determination of the change in molar enthalpy (ΔH)[134] and can detect changes in thermal power in the range of microwatts.[133]

ITC has been used to investigate interactions between varying molecular interactions including protein-ligand interaction, protein-oligonucleotide interaction, protein-carbohydrate interactions, lipid systems, carbohydrate-ligand interaction and protein folding. It has also gained wide interest for different food applications. There is however little data on using ITC to study amylose-inclusion complexes.

Silverio et al.[133] employed microcalorimetric titration in the investigation of starch retrogradation that occurs in the first 24hr. Purified amylose and amylopectin from corn, and native starches from wheat, potato, maize, waxy and amylomaize were investigated with or without the addition of sodium dodecylsulfate (SDS) or 1-monlauroylrac-glycerol (GML). Net exothermic heat of reaction for retrogradation decreased on addition of the lipids but this varied depending on the amylose content, botanical source, and type of lipid added. ITC was also able to show interactions between waxy maize and the added lipids. Another study that employed the use of ITC was by Kubik and Wulff.[125] which was discussed earlier. The effect of chemical modification of amylose on amylose inclusion complex was examined with the aid of ITC.

To further understand the amylose-lipid interaction, this sensitive method (ITC) can provide information regarding the interactions occurring at a micro level. It can also provide more information regarding the effects of varying chain lengths of amylose and fatty acid on the degree of interaction between the two molecules.

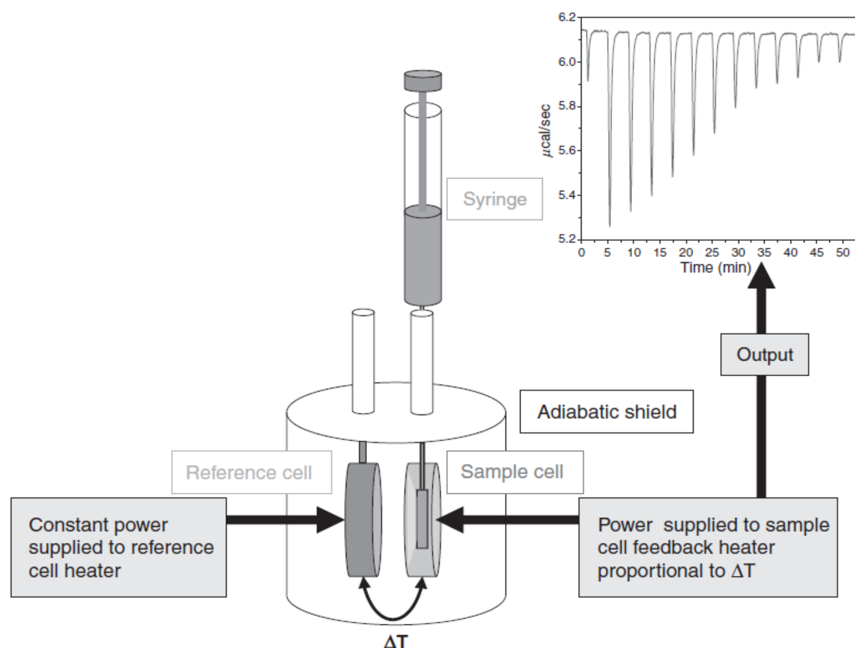

Figure 13. Representative diagram of a typical power compensation ITC.[134]

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III. CHAPTER 1: Effects of Botanical Source and Enzymatic Modifications on the Starch-Stearic Acid Complex Formation

ABSTRACT

Enzymatic modification of starch may create more favorable starch chains that could enhance starch-inclusion complexation. Starches from different botanical sources, including potato, common corn and Hylon VII, were modified enzymatically using isoamylase or combined with β-amylase prior to complexing with stearic acid, and starches and their complexes were characterized. Debranching significantly increased iodine affinity (IA) of potato and common corn, but had no effect on Hylon VII starch; the additional β-amylase treatment further increased IA of potato and common corn starches, but decreased that of Hylon VII. The highest amount of stearic acid (55.9 mg/g) was complexed by the debranched and β-amylase-treated potato starch. In general, the IA values of complexes were positively correlated with the amount of stearic acid measured by gas chromatography (GC). All starch complexes displayed a mixture of the B- and V-type X-ray diffraction patterns, with the debranched and β-amylase treated starch complexes exhibiting more of the V-type pattern. These results indicate that the additional β-amylase treatment significantly increased complexation between starch and stearic acid for debranched potato and common corn starches, but debranching alone was sufficient to increase complexation for Hylon VII starch under the present experimental conditions.

KEYWORDS: β-amylase, starch inclusion complexes, stearic acid, iodine affinity, X-ray diffraction

INTRODUCTION

Starch is a homoglucan of a similar backbone of repeating α-D-glucose units linked by α-D-(1→4) glucosidic linkages with branching points linked by α-D-(1→6) linkages. Starch consists of two main components, i.e. amylose of an essentially linear molecule with few branches, and amylopectin of a highly branched structure. Nevertheless, the characteristics of starch from different plant sources vary and are affected by the proportion and structure of amylose and amylopectin.[1,2] Native starches from different sources exhibit three distinct wide-angle powder X-ray diffraction patterns, including the A-, B- and C-type.[3] Cereal starches such as maize and rice exhibit the A-type polymorph; tuber starches like potato display the B-type; the C-type is found in bean or root starches.[4] The V-type polymorph is composed of a single amylose helix with a ligand included in its cavity and is formed primarily during complexation reaction with amylose.[5] In dilute solution, linear starch chains assume a helical conformation with a hydrophilic exterior and a hydrophobic cavity, which enables its interactions with hydrophobic compounds to form starch-inclusion complexes that usually exhibit the V-type polymorph.

Starch inclusion complexes have been proposed to function as carriers for delivering and protecting bioactive molecules from degradation[6,7] and for stabilizing volatile compounds.[8] Inclusion complexes involving starch and various molecules such as lipids,[9-11] flavors[8,12] and bioactive compounds[6,13,14] have been studied. The formation of inclusion complexes between amylose and fatty acids has been reported to be affected by many factors, such as amylose chain length,[15-18] lipid structure and chain length,[7,19-20] reaction temperature,[21-22] reaction pH[24] and complexing solvent.[25] The stability of amylose-fatty acid complexes was reported to decrease with unsaturation of the fatty acids[19,25] but increase with increasing amylose chain length up to

degree of polymerization (DP) 400, after which conformation disorders and crystal faults may arise for longer chains.[11]

Debranching has been employed to improve starch complexing capability.[26,27] Recently, Zhang et al.[28] debranched high-amylose (70% amylose) maize starch using pullulanase for varying times (0, 2, 6, 8, 12 and 24 h), and the resultant debranched starches were complexed with lauric acid. They reported that starch debranched for 24 h yielded the highest amount of complexed lauric acid, suggesting that prolonged debranching could improve the formation of starch-lipid complexes.

The objective of this study was to determine the effect of debranching in combination with an additional β-amylase treatment on starches from three sources, potato (~ 21% amylose), common corn (~ 27% amylose), and high amylose (~ 70% amylose) corn for the formation of starch-stearic acid (C18:0) complex. It was hypothesized that an additional β-amylase treatment on debranched starch could improve the complexation yield of some starches by producing starch chains with favorable lengths required for complexation with stearic acid. The complexation yields, iodine affinity, melting properties and X-ray diffraction patterns of the insoluble complexes formed were characterized.

MATERIALS AND METHODS

Materials. Potato starch was obtained from Penford Food Ingredients (Centennial, CO, USA). Common corn and Hylon VII (~70% amylose) starches were obtained from Ingredion Inc. (Bridgewater, NJ, USA) and defatted with 85% methanol prior to the complexation reaction to remove naturally present lipids. Isoamylase from *Pseudomonas sp* (specific activity 280 units/mg protein), pullulanase from *Klebsiella planticola* (specific activity 34 units/mg protein) and β-

amylase from *Bacillus cereus* (specific activity 2660 units/mg protein) were purchased from Megazyme International Ireland Ltd. (Wicklow, Ireland). Stearic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of ACS grade.

Enzymatic Modification of Starch

Debranching. Potato Starch (15 g wet basis) or other starches (common corn or Hylon VII) (20 g wet basis) was mixed with 400 mL water (3.75% or 5% w/v, respectively) and gelatinized in a boiling water bath for 1 h with constant shaking, and then the mixture was equilibrated to 45 °C and adjusted to pH 3.5 with 0.5 M HCl. To the starch solution, isoamylase (0.5% v/w starch db) was added and then incubated at 45 °C with constant stirring for 48 h. The starch was recovered with 4-fold volume of pure ethanol, centrifuged at 7000*g* for 10 min, dried at 40 °C for 48 h, and milled using a UDY cyclone mill (UDY Corp., Ft. Collins, CO, USA) fitted with a 0.5-mm screen.

β-Amylase Treatment. An additional β-amylase hydrolysis was used in some of the debranched starch to reduce the molecular size as described by Arijaje et al.[29] After the debranching, the starch slurry was adjusted to pH 6.5 with 0.5 M NaOH and incubated with 75 μL of β-amylase at 40 °C for 4 h. The enzymatic reaction was terminated by boiling for 15 min, and the β-amylase-treated starch was recovered as previously described for the debranched starches.

Characterization of Starch Structure. The molecular size distributions of debranched starch without or with the β-amylase treatment were characterized using a high-performance size exclusion chromatography (HPSEC) system (Waters Corp., Milford, MA, USA) as described by Arijaje and Wang.[30] Starch (10 mg) was dissolved in 5 mL of 90% DMSO, boiled for 1 h, and filtered through a 5.0-μm filter prior to injection into the HPSEC system. The HPSEC system consisted of an ultrahydrogel guard column and an Ultrahydrogel 250 column (Waters Corp.,

Milford, MA, USA), a 200 µL injector valve (model 7725i, Rheodyne, Cotati, CA, USA), an inline degasser, a model 515 HPLC pump, and a model 2414 refractive index detector. The mobile phase of 0.1 M sodium nitrate with 0.02% sodium azide was eluted at a flow rate of 0.6 mL/min. The temperature of column was maintained at 55ºC and the detector at 40ºC. Dextran standards of molecular weight of 5,200, 11,600, 23,800, 48,600, 273,000 and 410,000 g/mole from Waters Corp. (Milford, MA, USA) and 1,050,000 g/mole from Sigma-Aldrich (St. Louis, MO, USA) were used to establish the calibration curve.

Complexation of Starch and Stearic Acid. The starch solution (3.75 % potato or 5 % common corn and Hylon VII, w/v), debranched starch without or with the β-amylase treatment, was adjusted to pH 7.0, equilibrated to 80 °C, and added with 1 g of stearic acid (dissolved in 25 mL warm ethanol). The mixture was stirred continuously and maintained at 80 °C for 30 min to ensure sufficient mixing of starch and stearic acid, and then reduced to and maintained at 45 °C overnight with continuous stirring. The resulting starch-stearic acid mixture was centrifuged at 7000*g* for 15 min, and the precipitate was recovered. The uncomplexed stearic acid was removed by rotating the precipitates with 95% ethanol using a labquake shaker rotisserie (Barnstead/Thermolyne, Dubuque, IA, USA) at room temperature for 2 h, centrifuged at 7000*g* for 15 min, dried at 40 °C for 48 h, milled using a mortar and pestle, sieved through a 250-µm sieve, and stored for further analysis. All samples were replicated.

Iodine Affinity of Starches and Complexes. The iodine affinity (IA) of starches and complexes were determined by potentiometric titration according to Schoch.[31] Each sample (100 mg) was dissolved in 1 mL of water and 5 mL of 1 M KOH and placed in a refrigerator for 30 min with intermittent mixing. The sample was neutralized with 0.5 M HCl, and then 10 mL of KCl and water was added to achieve a total sample weight of 100.9 g. The solution was titrated

against 0.2 mg/mL standardized iodine solution using a potentiometer (Orion 420 plus, Thermo Electron Corp., Beverly, MA) by recording the EMF in millivolts. The bound iodine is the difference between the total iodine added and the free iodine from the blank titration. The IA was calculated using the formula below. The apparent amylose content was then determined by comparing against the typical IA value of purified linear fraction of the type of starch used. The typical values of corn and potato amyloses are 19.0% and 19.9%, respectively. Complexation capability was determined as the difference in IA between starches and their starch complexes.

$$\% \text{ Iodine affinity (IA)} = \frac{\text{mg of bound iodine at zero intercept x 100}}{\text{mg of sample weight (dry basis)}}$$

Stearic Acid Analysis. The complexed stearic acid was analyzed according to the method described by Arijaje et al.[29] Complex (100 mg) was mixed with 10 mL of 1 M HCl and incubated in a boiling water bath with continuous stirring for 1 h. The mixture was cooled and added with 10 mL hexane, and rotated on the rotary shaker for 2 h. Two mL of the recovered hexane layer containing the extracted stearic acid was added with 1 mL of boron trifluoride methanol to convert stearic acid to stearic acid methyl esters. An internal standard of methyl heptadecanoate (~1 mg) was subsequently added to all samples. The stearic acid methyl esters was injected into a gas chromatographer (GC) (GC-2010, Shimadzu, Kyoto, Japan) equipped with a BP 21 capillary column (30 m × 0.25 mm i.d.; SGE Inc., Austin, TX) with a flame ionization detector (FID), and responses were collected by Shimadzu GCsolution Workstation 2.3 (Kyoto, Japan). The injection port and detector temperatures were set at 220 °C and 230 °C, respectively. The column oven temperature was equilibrated at 100 °C for 1 min, ramped up at 15 °C /min to 160 °C, again ramped up at 5 °C /min to 200 °C and maintained at 200 °C for 10 min. The flow rate of the carrier gas (helium) was 30 mL/min. The concentration of stearic acid was determined

from a standard curve prepared by using stearic acid methyl ester solution (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL) containing the internal standard of methyl heptadecanoate (0.5 mg/mL).

Physicochemical Properties. A diffractometer (PW1830 MPD, Philips, Almelo, The Netherlands) was used to determine the powder X-ray diffraction pattern of starch and starch complexes. The generator voltage was set at 45 kV and the current at 40 mA. The sample was scanned over the 2θ angular range from 5° to 35° with a step size of 0.02° and time of 1 s per step.

The thermal properties of all complexes were measured using a differential scanning calorimeter (DSC, Pyris-Diamond, PerkinElmer, Shelton, CT, USA). Approximately 8 mg of starches and complexes were weighed into stainless steel pans, 16 µL of distilled water was added with the aid of a micro syringe, and the pans were hermetically sealed. The samples were equilibrated overnight at room temperature before scanning from 25 to 180 °C at 10 °C/min. The onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c) and enthalpy (ΔH) of the endotherms were calculated using the Pyris data analysis software.

Statistical Analysis. All experiments were conducted at least in duplicate and analyzed using JMP software (SAS Institute Inc., Cary, NC, USA) and the means of the data were compared using Tukey's honestly significant differences (HSD) test.

RESULTS AND DISCUSSION

Molecular Size Distribution of Starches. The standard curve used to estimate the degree of polymerization of debranched starches without or with the β-amylase treatment is displayed in Figure 1a, and the corresponding chromatograms of all starches are presented in Figure 1b.

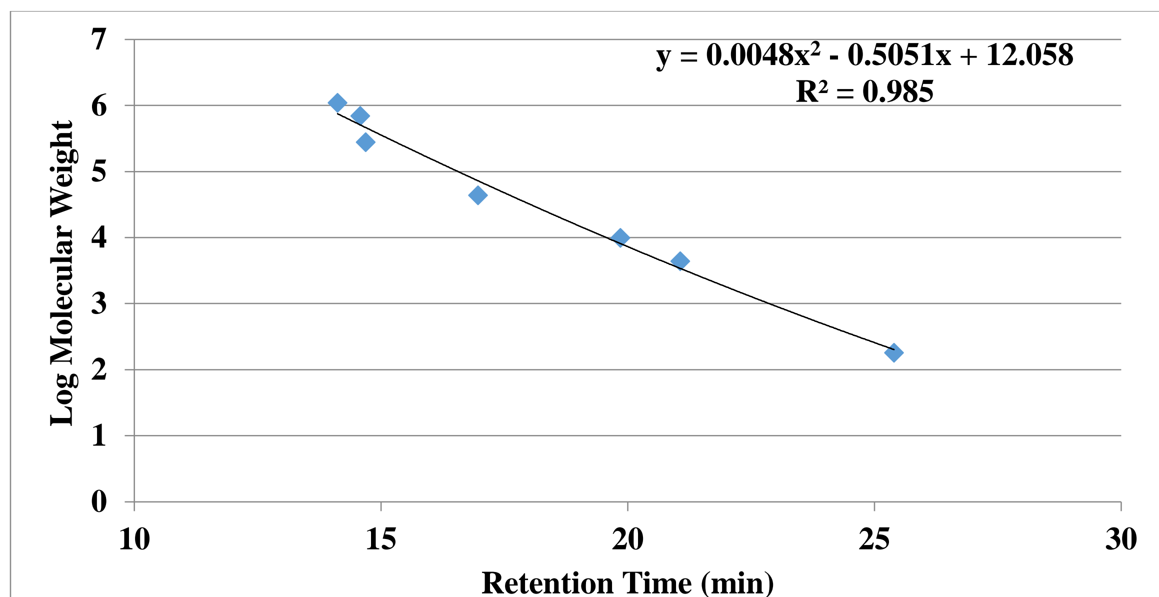

Figure 1a. Standard curve used to estimate the degree of polymerization of debranched starches without or with the β-amylase treatment.

The amylose and amylopectin fractions were divided at the minimum points of the profile, which was at a retention time (RT) of 17.04 min for potato and 18.44 min for both common corn and Hylon VII starch. For the debranched-only starch, the proportion of amylose was 21.8% for potato, 23.9% for common corn, and 61.6% for Hylon VII starch, which were similar to the amylose contents usually associated with these commercial starches (Table 1).

The debranched potato starch exhibited 4 peaks with peak RTs at 12.83, 14.23, 19.45 and 21.18 min. The debranched common corn starch had 2 peaks with peak RTs at 15.01 and 21.46. Hylon VII starch displayed a very broad profile with peak RTs at 15.93 and 20.93 min. The peak of debranched potato starch with a peak RT 12.8 min signified the presence of a high molecular weight amylose, which was not observed for common corn and Hylon VII starch. Potato amylose is known to have a higher molecular weight with a DP range of 840-21800[32] compared with

common corn of DP 400-14700[33] and Hylon VII of DP 270-8940,[34] and the present results confirm those reports.

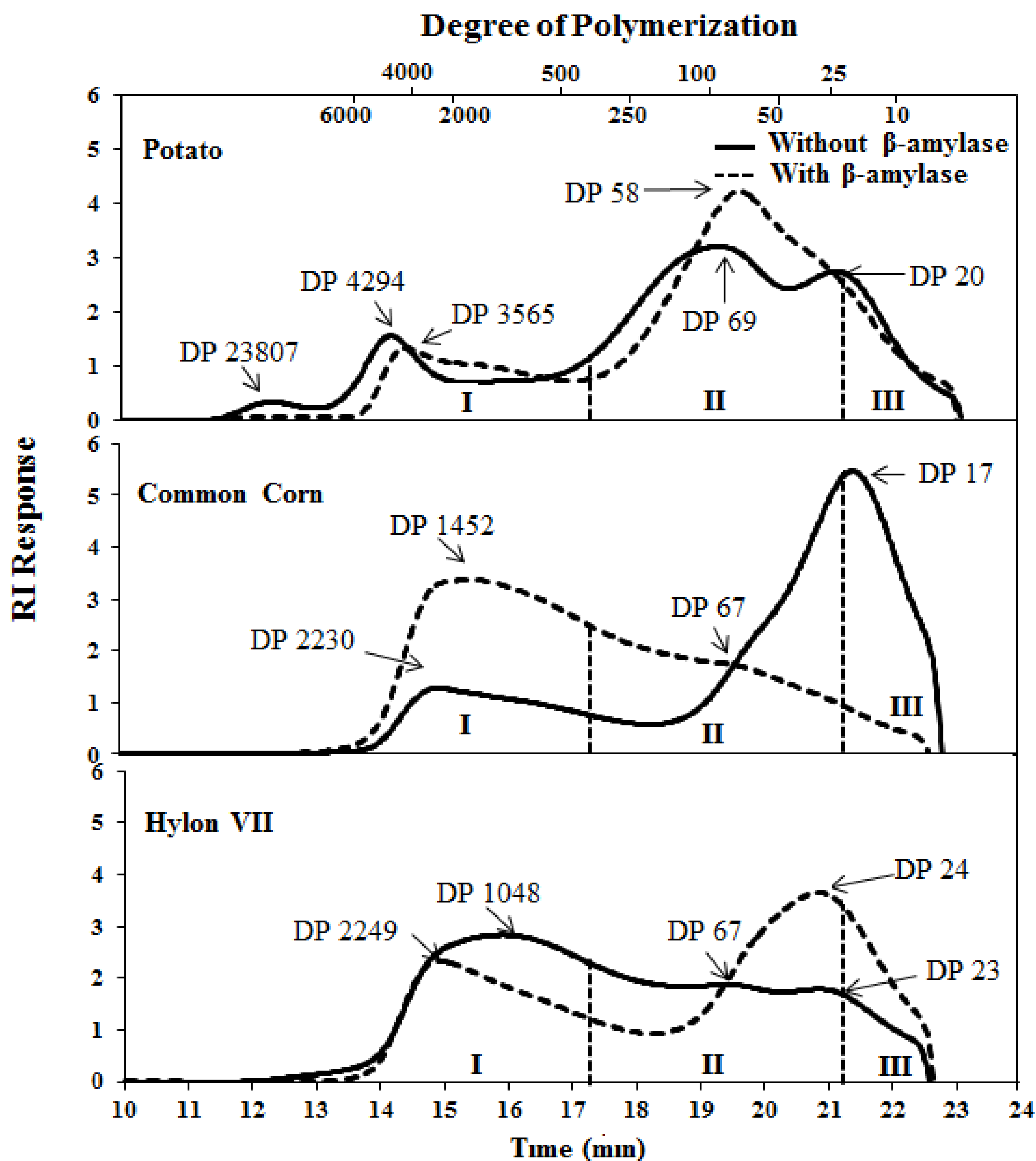

Figure 1b. Normalized size-exclusion chromatograms of debranched starches without or with the β-amylase treatment.

Table 1. Percentages of Starch Fractions for Native, Debranched-only and Debranched and β-amylase-Treated Starches Calculated from their Molecular Size Distributions.[a]

starch	β-amylase treatment	starch fractions				
		amylose	AP	I (DP > 400)	II (DP 20-400)	III (DP < 20)
potato	No	21.8±0.2e	78.3±0.6b	22.3±0.8d	60.8±1.1b	16.9±0.3c
	Yes	13.7±0.4f	86.3±1.6a	14.2±0.4f	68.7±0.9a	17.1±0.2c
common corn	No	23.9±0.1d	76.2±0.5c	18.8±0.2e	46.1±0.3d	35.0±0.5a
	Yes	71.3±0.7a	28.7±0.3f	53.7±0.9a	42.3±0.5e	4.0±0.2e
Hylon VII	no	61.6±0.9b	38.5±0.6e	45.8±0.7b	45.3±0.4d	9.0±0.8d
	yes	40.8±1.0c	59.2±0.0d	32.5±0.1c	49.3±0.3c	18.2±0.4b

[a]Data of at least two measurements with standard deviation. Means in a column not sharing the same letter are significantly different based on Tukey's honestly significant difference test ($p < 0.05$).

The starch chains with a RT shorter than 17.1 min, which corresponded to DP > 400, were proposed to be too long to participate in stable complex formation for all starches.[11] This study by Gelders et al.[11] had examined only amylose with peak DP 20, DP 60, DP 400 and DP 950. On the other hand, starch chains with DP < 20 were too short to form complexes with lipids.[16] Thus, the optimum chain length for starch complexation was suggested to be between DP 20 and 400, and therefore starch chains were divided into three fractions, DP > 400 (I), 20 ≤ DP ≤ 400 (II) and DP < 20 (III) to calculate the proportions of each fractions (Table 1). Fraction (Fr.) II, the proportion of starch chains proposed to favor for complexing with stearic acid, was found to be the predominant fraction in all starches, with potato starch displaying the highest proportion, followed by Hylon VII and then common corn starch before the β-amylase treatment. Common corn starch had the highest proportion of Fr. III, whereas Hylon VII had the highest

proportion of Fr. I. Both potato starch and Hylon VII comprised over 80% of Fr. I and II, which agree with Hizukuri et al.[35] that their B-type X-ray diffraction pattern has been associated with their longer average chain lengths. In contrast, common corn starch has the A-type X-ray diffraction pattern and a shorter average chain length.[35]

The molecular weight distributions significantly changed after the β-amylase treatment, especially for common corn and Hylon VII starches. The amylose fraction significantly decreased for potato (13.7%) and Hylon VII (40.8%) starches, but significantly increased for common corn starch (71.3%), which was correlated with the change in Fr. I. he decrease in amylose fraction and Fr. I for potato and Hylon VII starches was attributed to the hydrolysis of longer amylose and amylopectin chains, which caused a shift to a lower DP and reduced the proportion of the amylose fraction. The drastic increase in Fr. I of common corn starch (35 percentage points) after the β-amylase treatment was proposed to be due to the extensive hydrolysis of amylopectin short chains by β-amylase, thereby increasing the proportion of amylose relative to the amylopectin.

The Fr. II of potato and Hylon VII starches increased 8 and 4 percentage points, respectively, whereas that of common corn starch decreased 4 points. The increase of Fr. II in potato and Hylon VII starches was attributed to their higher average chain lengths from a greater proportion of long chains, which became more uniformly distributed after the β-amylase treatment. Hylon VII and potato starch contain a high percentage of short chain amylose[36] and long chain amylose, respectively,[37] which may also contribute to their increase in Fr. II after the β-amylase treatment. In contrast, common corn starch has a higher percentage of short amylopectin chains,[35] which were hydrolyzed and only some long amylopectin chains remained.

Iodine Affinity and Apparent Amylose Content of Starches and Complexes. The IA values and their corresponding apparent amylose contents of native starches and debranched starches without and with the β-amylase treatment are presented in Table 2. The amylose contents of native starches were similar to the amylose fractions as determined by their respective molecular size distributions of debranched starches using HPSEC (Table 1).

Table 2. Iodine Affinity and Apparent Amylose Content[b] for Native and Debranched Starches Without and With the β-amylase Treatment and their Complexes.[a]

	starches		starch complexes	
starch	iodine affinity	% apparent amylose content[b]	iodine affinity	% apparent amylose content
potato				
native	4.8±0.4f	24.3±1.9f	N/A[c]	N/A
debranched-only	9.9±0.1d	49.6±0.3d	6.4±0.2b	32.8±0.0b
debranched with β-amylase	15.8±0.2a	83.3±0.8a	4.6±0.4c	23.0±2.0c
common corn				
native	4.6±0.0f	24.4±0.1f	N/A	N/A
debranched-only	7.4±0.3e	39.0±1.4e	5.6±0.2b	29.5±1.2b
debranched with β-amylase	14.7±0.3b	77.1±1.8b	4.5±0.0c	23.8±0.1c
Hylon VII				
native	12.4±0.2c	65.4±1.0c	N/A	N/A
debranched-only	12.2±0.1c	64.2±0.5c	8.3±0.0a	43.6±0.2a
debranched with β-amylase	10.0±0.3d	52.9±1.7d	8.8±0.2a	46.0±0.9a

[a]Data of at least two measurements with standard deviation. Means in a column not sharing the same letter are significantly different based on Tukey's honestly significant difference test ($p < 0.05$).
[b]Apparent amylose content was determined by comparing against the typical iodine affinity value of purified linear fraction of the corn and potato amyloses, which are 19.0% and 19.9%, respectively.
[c] N/A, not applicable

The debranching increased the IA and apparent amylose content of potato and common corn starches significantly but had no significant impact on those of Hylon VII starch. The increase in IA and apparent amylose content in potato and common corn starches was ascribed to the long amylopectin chains that became available to complex with iodine after debranching, and their different increases indicate that potato starch had a greater proportion of long amylopectin chains than common corn starch. The average amylopectin chains in native Hylon VII was reported to be 30.9,[35] which was sufficient long to complex with iodine even before debranching, therefore there was no noticeable difference in the apparent amylose content between native and debranched Hylon VII starch.

When the β-amylase treatment was applied, there was a further significant increase in the apparent amylose content from 49.6 to 88.3% for potato starch and from 39.0 to 77.1% for common corn starch; however the apparent amylose content of Hylon VII was decreased significantly from 64.1 to 52.9%. The increase in IA for potato and common corn starch was because the β-amylase treatment helped to reduce the chain length of starch chains to the favorable length required for complexation, which was achieved by hydrolysis of short amylopectin chains to result in more chains with favorable DP for complexation. This is supported by the HPSEC results, where the proportion of long chains (Frs. I and II) unchanged or increased for potato and common corn starches, but that of Hylon VII decreased. The Hylon VII results indicate that Hylon VII amylopectin long chains took part in the IA measurements, but subsequently became too short after the β-amylase treatment to form complexes with iodine. These results also corroborate well with the HPSEC results where Hylon VII starch exhibited an increase in the proportion of shorter chains (Fr. III) (Table 1) after the β-amylase treatment. The present results suggest that debranching of potato and common corn starches can improve their

capability to form complexes by reducing the DP of amylose and amylopectin chains to an optimum DP range.

Following the enzymatic treatments of starches, starch-stearic acid complexes were prepared and recovered, and their IAs was measured to estimate the extent of complex formation based on the reduction in IA after stearic acid complexation. If starch helix was occupied by stearic acid, it would not be capable of complexing with iodine because iodine and fatty acids occupied the same location in the starch helix.[38] The IA for debranched starch-stearic acid complexes were 6.4, 5.6 and 8.3 for potato, common corn and Hylon VII, respectively, which corresponds to a complexation yield of 19.8, 9.5 and 20.6% respectively. These results showed that for the debranched starches, only common corn starch showed a low complexation yield, and agree with the HPSEC results that debranched common corn starch consisted of a high percentage of short chains that were ineffective for complexation.

With the additional β-treatment, the IA of complexes was significantly reduced when compared with the starches prior to complexation, from 15.8 to 4.6 for potato, from 14.7 to 4.5 for common corn, and from 10.0 to 8.8 for Hylon VII starch. As previously discussed, the reduction in IA of starch complexes corresponded to the increase in complexation with stearic acid. These results therefore show that complexation yield was increased for potato and common corn starches but was reduced for Hylon VII after the β-amylase treatment. Although the present results support previous studies[11,16] that starch chains in Fr. II are responsible for the complexation reaction, these results also suggest that starch chains with DP > 400 may participate in complexation with stearic acid. The IA of debranched common corn starch decreased significantly after the β-amylase treatment, which indicated an increase in complexation and was correlated with an increase in the proportions of Fr. I (Table 1).

Nevertheless, the β-amylase-treated Hylon VII starch complexes showed a high proportion of starch chains in Fr. II, but a low complexation with stearic acid compared with potato and common corn starches, which was suspected to be due to an increased Fr. III that was not favorable for complexation.

Overall, when the IA values of complexes from both the debranched without and with the β-amylase treatment were correlated with the HPSEC results, the present results suggest that complexation of stearic acid occurred mainly with starch chains in Fr. II, and to a much less extent with starch chains in Fr. I .

Complex Recovery and Stearic Acid Content. The recovery of starch-stearic acid complexes were obtained by dividing the weight of recovered insoluble complex by the sum of initial starch and stearic acid weight. The recovery of the debranched-only starch complexes followed the order Hylon VII > common corn > potato. The additional β-amylase treatment had no significant effect on the recovery of starch complexes from common corn and Hylon VII starch, but it significantly increased the recovery for the potato starch complexes. It is possible that the percentage of phosphate monoesters (0.09% dry starch basis)[39,40] naturally present in potato starch was increased by the β-amylase treatment, which stabilized starch chains and encouraged complexation. The amounts of stearic acid recovered and measured by GC from all starch complexes are listed in Table 3. Within the debranched starch, the included stearic acid was in the order of Hylon VII starch > potato starch > common corn starch.

Table 3. Complex Recovery and Stearic Acid Content[a] Recovered from all Debranched Starch-Stearic Acid Complexes.

starch-stearic acid complex	β-amylase treatment	recovery (g/g)	stearic acid[b] in complex (mg/g)
potato	no	0.59±0.0c	25.1±0.4d
	yes	0.76±0.4b	55.9±2.1a
common corn	no	0.67±0.1c	13.7±0.9e
	yes	0.63±0.1c	33.5±0.5c
Hylon VII	no	0.93±0.1a	37.3±0.4b
	yes	0.95±0.2a	33.5±0.7c

[a]Data of at least two measurements with standard deviation. Means in a column not sharing the same letter are significantly different based on Tukey's honestly significant difference test ($p < 0.05$).
[b]Stearic acid measured by GC.

When the additional β-amylase treatment was applied, the amount of included stearic acid increased significantly for potato and common corn starch complexes, but it decreased slightly for Hylon VII starch complexes. The β-amylase-treated potato starch complex included the highest amount of stearic acid (55.9 mg), and there was no significant difference between the amount of complexed stearic acid for the β-amylase-treated common corn and Hylon VII starch complex. These results correlated well with the previous results from the IA and apparent amylose content (Table 2), in which the β-amylolysis of starch significantly improved complex formation with stearic acid for potato and common corn starches, but decreased for Hylon VII starch. These results also suggest that although stearic acid and iodine may occupy the same position in starch helix,[38] their individual interactions with the starch helix differ because of the difference in their chemical structures and molecular weight.

When the amounts of complexed stearic acid was compared with starch chain fractions, the results from the present study are in agreement with previous studies[11,16] and IA results that starch chains $20 \leq DP \leq 400$ are effective for starch complexation with stearic acid, except for the β-amylase-treated Hylon VII starch, which was discussed earlier and was attributed to the increase in very short starch chains (Fr. III) that was too short to form complexes. Starch chains with DP > 400 (Fr. I) were also actively participating in complex formation.

Characterization of Starch-Stearic Acid Complexes

X-ray Diffraction Pattern. The X-ray diffraction patterns of native ungelatinized, debranched starches without and with the β-amylase treatment and their starch-stearic acid complexes are displayed in Figure 2. Native and debranched potato and Hylon VII starch displayed the B-type X-ray diffraction pattern with peaks at $2\theta = 5.6$, 15.3º, 17.2º, 19.7º, 23.4º (Figure 2), whereas native and debranched common corn displayed the A-type X-ray pattern with characteristic peaks at $2\theta = 10º$, 11.4º, 15.2º, 17.2º, 18.2 º, and 23.1º.These results are in agreement with previous studies where tuber starches and amylomaize starch have been reported to have the B-type pattern[35,41] and common corn the A-type pattern.[42] Hizukuri et al.[35] reported that the A-type starches have shorter average chain lengths (DP < 19.7) compared with the B-type starches (DP > 21.6) and crystallize to display the A- and B-type patterns, respectively. This may explain why native ungelatinized Hylon VII starch displayed the B-type pattern. In addition, native ungelatinized and debranched Hylon VII starch displayed an additional peak at $2\theta = 20º$, which signifies the presence of naturally present amylose-lipid complexes.[41] This suggests that the naturally present lipids were not completely removed in Hylon VII starch during the defatting process.

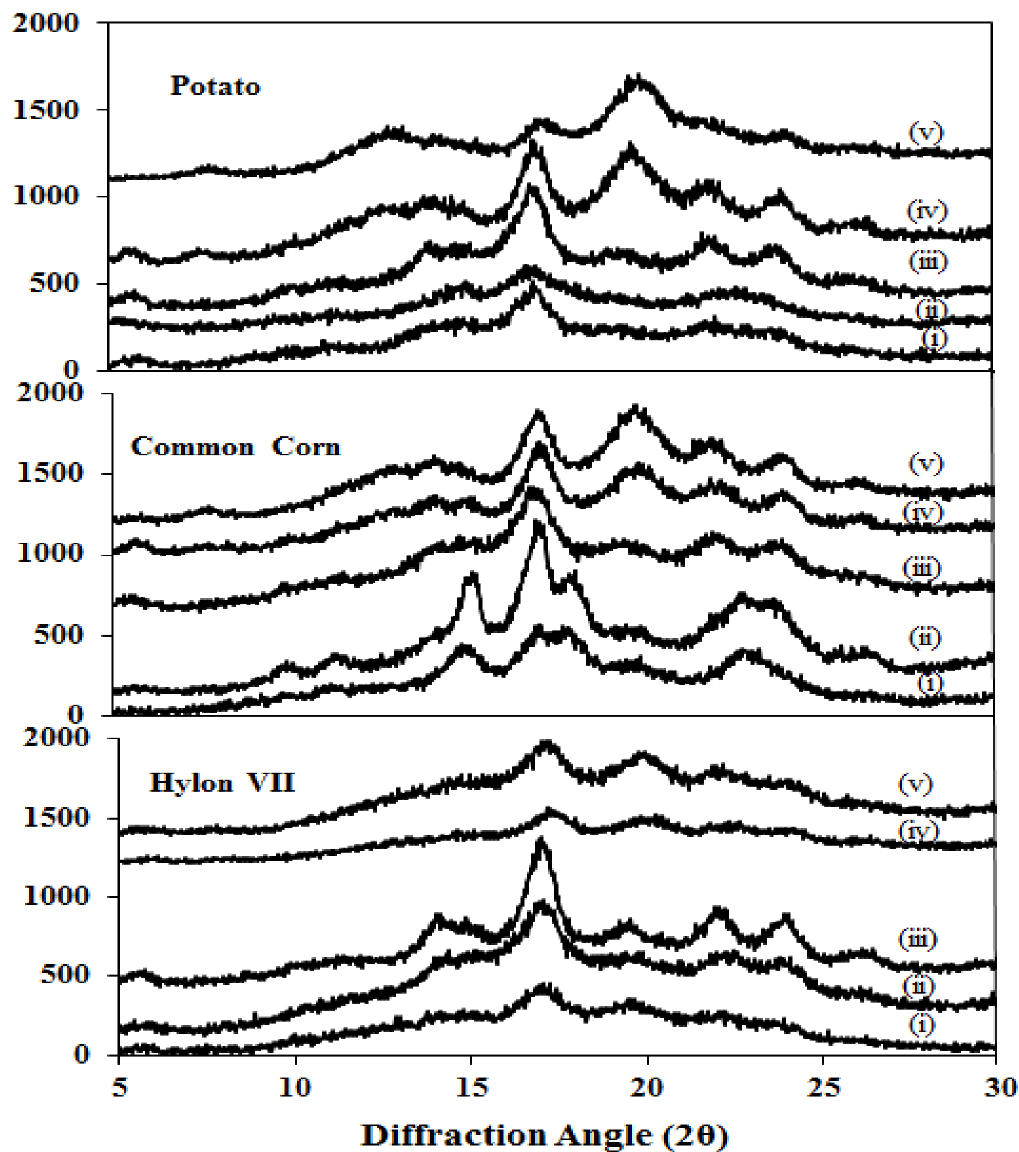

Figure 2. Normalized X-ray diffractograms of native and debranched potato, common corn and Hylon VII starches and their starch complexes. (i) native ungelatized starch; (ii) debranched starch; (iii) debranched and β-amylase-treated starch; (iv) debranched starch-stearic acid complex; (v) debranched and β-amylase-treated starch-stearic acid complex.

The XRD patterns displayed by the starches were supported by the HPSEC chromatograms (Figure 1b). The A-type pattern exhibited by common corn starch was supported

by the HPSEC results, which showed the presence of a high proportion of amylopectin short chains that have been implicated in the formation of the A-type crystalline pattern. The peak intensities increased with debranching for common corn and Hylon VII starches, and decreased slightly for potato starch, but all starches retained the same XRD pattern before and after the debranching process, suggesting that the debranching treatment did not affect how starch chains arranged themselves to form the crystalline structure.

When the β-amylase treatment was incorporated to the debranched starches, all three resultant starches displayed the B-type pattern. Debranched common corn starch with the A-type pattern was transformed to the B-type pattern, signifying a change in crystalline structure. The transformation of the XRD pattern of common corn starch is supported by the HPSEC results, in which the proportion of short chains (Fr. III) significantly decreased after the β-amylase treatment and the significant increase in Fr. I may contribute to the formation of the B-type pattern.

The X-ray patterns of all starch-stearic acid complexes were a mixture of the B- and V-type pattern (peaks at $2\theta = 7.6^{o}$, 12.9^{o} and 20°), indicating the presence of retrograded starch chains and starch-stearic acid complexes. Zhang et al.[28] also observed a mixture of the B- and V-type pattern in the debranched Hylon V-lauric acid complex. When an additional β-amylase treatment was applied to the debranched starches, the intensity of the V-type pattern was increased, supporting the previous the IA and GC results that the debranched starches formed starch-stearic acid complexes, and the additional β-amylase treatment further encouraged complex formation. The intensity of the B-type pattern, particularly the peak at $2\theta = 17.2^{o}$, increased significantly for the β-amylase-treated Hylon VII complexes, but decreased for potato

and common corn starches, which may explain the negative effect of the β-amylase treatment on complexation with stearic acid for debranched Hylon VII starch.

Thermal properties of Starch and Starch-Stearic acid Complexes The melting properties of starch-stearic acid complexes as measured by DSC are presented in Table 4. The peak melting temperature of complexes (T_p) without and with the β-amylase treatment ranged from 105.8 to 108.8 ºC for all starches, depicting the melting of the type I starch-stearic acid complex[22], which has been reported to dissociate between 95 and 105 ºC.[25] Within each starch, the additional β-amylase treatment only slightly increased the T_p and T_c, but it significantly increased the enthalpy value. The increase in enthalpy values might indicate more complex formation, as supported by the GC results (Table 3), and/or more energy required to melt these complexes due to an increase in their crystallinity, as supported by the X-ray results (Figure 2).

Table 4. Melting Temperatures and Enthalpies of Debranched Starch-Stearic Acid Complexes[a].

starch	β-amylase treatment	dissociation of starch-stearic acid complex			
		T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)
potato	no	94.5±0.5b	107.1±1.2a,b	116.9±1.2b,c	8.85±0.03b
	yes	96.9±0.1a	109.0±0.6a	118.6±0.0a,b	10.82±0.15a
common corn	no	94.9±0.1b	105.8±0.6c	117.8±0.1a,b	5.75±0.13c
	yes	94.9±0.4b	107.6±0.4a,b	119.1±0.3a	10.71±0.24a
Hylon VII	no	93.5±0.2b	108.8±0.3a	115.5±0.4c	2.30±0.08e
	yes	94.4±0.7b	108.8±0.0a	119.1±0.2a	4.88±0.12d

[a] Mean (standard deviation) of at least two measurements. Means in a column not sharing the same letter are significantly different based on Tukey's honestly significant difference test ($p < 0.05$). Melting temperatures: Onset, T_o; Peak, T_p; Conclusion, T_c; Enthalpy, ΔH.

CONCLUSIONS

In conclusion, the present study demonstrates that a combination of debranching and β-amylase treatment improved the amount of included stearic acid formation for both potato and common corn starches. Hylon VII starch, on the other hand, had the highest complex recovery among starches. The β-amylase treatment produced more starch chains with favorable chain lengths to form complex with stearic acid for potato and common corn starches. The debranched and β-amylase-treated potato starch included the highest amount of stearic acid. Starch chains with DP > 400 may also actively participate in complexing with stearic acid.

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APPENDIX

Authorship Statement for Chapter 1

I verify that Emily Arijaje is the first author and completed over 51% of the research work published in the following article that is included in her dissertation: Effects of Botanical Source and Enzymatic Modifications on the Starch-Stearic Acid Complex Formation.

Dr. Ya-Jane Wang

IV. CHAPTER 2: Complexation between Hexanoic Acid and Linear Starch Chains using Isothermal Titration Calorimetry - Chapter 2

ABSTRACT

There is limited information on the use of isothermal titration calorimetry (ITC) in understanding starch-fatty acid interactions. The effects of hexanoic acid concentration and temperature on the complexation of debranched waxy maize starch and potato amylose with hexanoic acid were investigated using ITC. The starch samples were characterized for their molecular size distribution and iodine affinity. Potato amylose and debranched waxy maize starch displayed a major peak degree of polymerization (DP) of 330 and 25, respectively and Iodine Affinity (IA) value was higher for the potato amylose. The titration data were fitted using a nonlinear least squares approach with one set of binding site model. Complexation was exothermic and spontaneous for all reactions based on changes in enthalpy free energy (ΔG). The binding affinity (K_a) of debranched waxy maize starch for hexanoic acid decreased with increasing temperature from 25 to 45ºC. The K_a and number of binding sites (n) for debranched waxy maize exceeded that of potato amylose, indicating a higher complexation of debranched waxy maize for hexanoic acid. The (n) and enthalpy of binding (ΔH) values for both starches remained independent of temperature change.

KEYWORDS: hexanoic acid, isothermal titration calorimetry, waxy maize starch, amylose.

INTRODUCTION

Most starches are composed of a mixture of two polydisperse and polymolecular polymers: amylose, an essentially linear polymer, and amylopectin, a highly branched polymer. Both are homopolymers of α-D-glucopyranose with the linear chains linked by α-D-(1→4) glucosidic linkages and the branching points by α-D-(1→6) linkages. Amylose forms a helical structure in solution with a hydrophobic cavity induced by the interaction with an hydrophobic compound with the polar hydroxyl groups aligned outwards and the less polar groups aligned inwards.[1] These inclusion complexes have been proposed as carriers for fatty acids[2], drugs[3] and other bioactive compounds.[4] Complexation between starch and fatty acids have been studied extensively with the aid of X-ray crystallography[5,6], differential scanning calorimetry (DSC)[7-9], and fourier transform infrared spectroscopy (FTIR)[10,11]. FTIR can also help to identify and quantify the particular compound in the complex because different compounds absorb and transmit infrared differently.

Isothermal titration calorimetry (ITC) has been used to characterize the thermodynamics of interaction occurring at a molecular level[12] over a range of temperatures.[13] First described by Hansen et al[14] and Christensen et al[15], ITC is an invaluable tool in understanding molecular interactions by measuring the heat output (exothermic reaction) or uptake (endothermic reaction) of a binding process, and thus, heat measured can be used to express the extent of interaction at equilibrium during titration.[13] Various types of molecular interactions such as protein-ligand interaction,[16-18] protein folding,[19] and protein-carbohydrate interaction[20,21] have been studied.

ITC was used to investigate the inclusion complex formation between synthetic amylose of varying degrees of polymerization (DP) of 9 – 1000 glucose units or amylopectin with 4-tert-butyl-phenol or SDS.[22] Amylopectin with chain length DP 13 and synthetic amylose with DP $\geq$ 9

formed complexes with SDS, but only amylose with DP ≥ 100 complexed with tert-butylphenol. Silverio et al[23] investigated the influence of surfactants, including sodium dodecylsulfate (SDS) or 1-monolauroylrac-glycerol (GML) on starch retrogradation using isothermal microcalorimetric titration. They reported a reduction in net exothermic heat of reaction with the addition of the surfactant, implying a decrease in retrogradation. In addition, the interactions with starch were affected by amylose content and botanical source of starch, as well as on the type of surfactants. ITC was also used to investigate maltodextrin/cyclodextrin and surfactant interactions.[24-27] Mun et al[27] reported (n) values of 2.5 and 5.3 for the binding of SDS and dodecyl trimethylammonium bromide (DTAB), respectively, to cycloamylose with an average molecular weight of 7500 g/mole. These results indicate that on average, two or three molecules of SDS and about five molecules of DTAB bound to one molecule of cycloamylose, respectively. However, limited information is available on the use of ITC in understanding starch-fatty acid complexation, and ITC may be able to provide more information that could enable the optimization of the starch-inclusion complex formation process.

The objective of the present study was to investigate the thermodynamics of complexation of linear starch chains from debranched waxy maize and potato amylose with varying concentrations of hexanoic acid (C:6) at two titration temperatures (25 and 45ºC). Hexanoic acid was used as a model compound for the starch-fatty acid complex formation study because it is hydrophobic to interact with the hydrophobic cavity of the starch helix to form complex yet relatively soluble in the buffer solution used for the ITC measurements. Waxy maize starch consists ~99% amylopectin and was used to create linear starch chains of lower DPs by debranching with isoamylase, whereas potato amylose represented long linear starch

chains. The results from this study could offer more information on the starch-inclusion complex formation at the molecular level in order to optimize the complexation reaction.

MATERIALS AND METHODS

Materials. Potato amylose and hexanoic acid were obtained from Sigma-Aldrich (St. Louis, MO) and used without further treatment. Waxy maize starch was obtained from Ingredion (Bridgewater, NJ, USA). Isoamylase (specific activity 59,000 units/mg protein) was purchased from Hayashibara Biochemical Laboratories Inc. (Okayama, Japan). All other chemicals were ACS grade.

Debranched waxy maize starch was prepared by gelatinizing waxy maize starch slurry (5 % w/v) in a boiling water bath for 1 h with constant stirring. Then the solution was equilibrated to 45°C, adjusted to pH 3.5 with 0.5 M HCl, added with 0.5% isoamylase (v/w starch dry weight) and then incubated at 45°C with constant stirring for 48 h. The debranched amylopectin was recovered with four-fold volume of pure ethanol, centrifuged, dried at 40°C for 48 h, and milled using a UDY cyclone mill (UDY Corporation, Ft. Collins, CO) fitted with a 0.5-mm screen.

Characterization of Starch Structure. The molecular-size distributions of potato amylose and debranched amylopectin from waxy maize starch were determined by a high-performance size-exclusion chromatography (HPSEC) system (Waters Corp., Milford, MA). Approximately 10 mg of starch was dissolved in 5 mL of 90% DMSO, boiled for 1 h, and filtered through a 5.0-µm filter prior to injection into the HPSEC system. The HPSEC system consisted of a guard column, two Shodex columns (OHpak SB-G, 6.0 × 500 (mm) i.d. × length), two Shodex columns (OHpak KB-804 and KB-802, both 8.0 × 300 (mm) i.d. × length), a 200 µL injector valve (model 7725i,

Rheodyne, Cotati, CA, USA), an inline degasser, a model 515 HPLC pump, and a model 2410 refractive index detector. The mobile phase of 0.1 M sodium nitrate with 0.02% sodium azide was eluted at a flow rate of 0.6 mL/min. The temperature of column was maintained at 60ºC and the detector at 40ºC. Dextran standards of molecular weight of 5,200, 11,600, 23,800, 48,600, 273,000, 410,000 and 872,300 g/mole from Waters Corp. (Milford, MA) and 1,185,000 g/mole from Sigma Aldrich (St. Louis, MO) were used to establish the calibration curve.

Physicochemical Properties. Iodine affinity (IA) was analyzed by potentiometric titration as described by Schoch.[28] Starch (~100 mg) was dissolved in 1 mL of water and 5 mL of 1 M KOH and placed in a refrigerator for 30 min with intermittent mixing. The sample was neutralized with 0.5 M HCl and 10 mL of 0.5 M potassium iodide (KI) was added. Water was then added to the solution to achieve a total weight of 100.9 g. The solution was titrated against ~0.2 mg/mL standardized iodine solution using a potentiometer (Orion 420 plus, Thermo Electron Corp., Beverly, MA) by recording the electromotive force (EMF) in millivolts from 230 to 285 mV. The bound iodine is the difference between the total iodine added and the free iodine from the blank titration. The IA was calculated using the formula below, and the apparent amylose content was determined by comparing the IA against the typical IA of purified amylose of corn and potato starch, which are 19.0% and 19.9%, respectively.

$$\%\ \text{Iodine affinity (IA)} = \frac{\text{mg of bound iodine at zero intercept} \times 100}{\text{mg of sample weight (dry basis)}}$$

Isothermal titration calorimetry

The complexation between amylose or debranched waxy maize starch and hexanoic acid was assessed by measuring the heat change that occurred during titration using a VP-ITC titration microcalorimeter (MicroCal Inc., Northhampton, MA). Prior to titration, amylose or debranched amylopectin (7.95 mg/mL) was solubilized in 10 mM tris buffer containing 10 mM NaCl at pH 7.4. Hexanoic acid (20, 25, 35 mM) was also solubilized in 10 mM tris buffer containing 10 mM NaCl at pH 7.4, and all solutions were equilibrated at 25°C and degassed under vacuum. The sample cell (1.4 mL) contained amylose and debranched amylopectin solution, the reference cell contained ultrapure water, and the injection port contained hexanoic acid concentration. Hexanoic acid (10 µL) was titrated into the sample cell every 6 min with a total of 35 injections. The resulting titration curves were corrected for ligand free buffer interactions and analyzed using the Origin ITC software by MicroCal Inc. Thermodynamic parameters characterizing the complexation of debranched waxy maize or potato amylose with varied concentrations of hexanoic acid were determined by running the ITC measurements at 25 and 45 ºC. The data obtained with these interactions was best fitted using a nonlinear least squares with one set of binding site model that generated the thermodynamic values of association constant (Ka) (where $K_a = 1/K_d$), number of binding sites (n), enthalpy of binding(ΔH) and entropy of binding (ΔS). The change in free energy (ΔG) for all reactions were calculated using the Gibb's free energy equation, $\Delta G = \Delta H - T\Delta S$, where T is the absolute temperature in Kelvin.

RESULTS AND DISCUSSION

Molecular size distribution of starch. The standard curve used to estimate the degree of polymerization for the debranched waxy maize starch and potato amylose is displayed in Figure 1a.

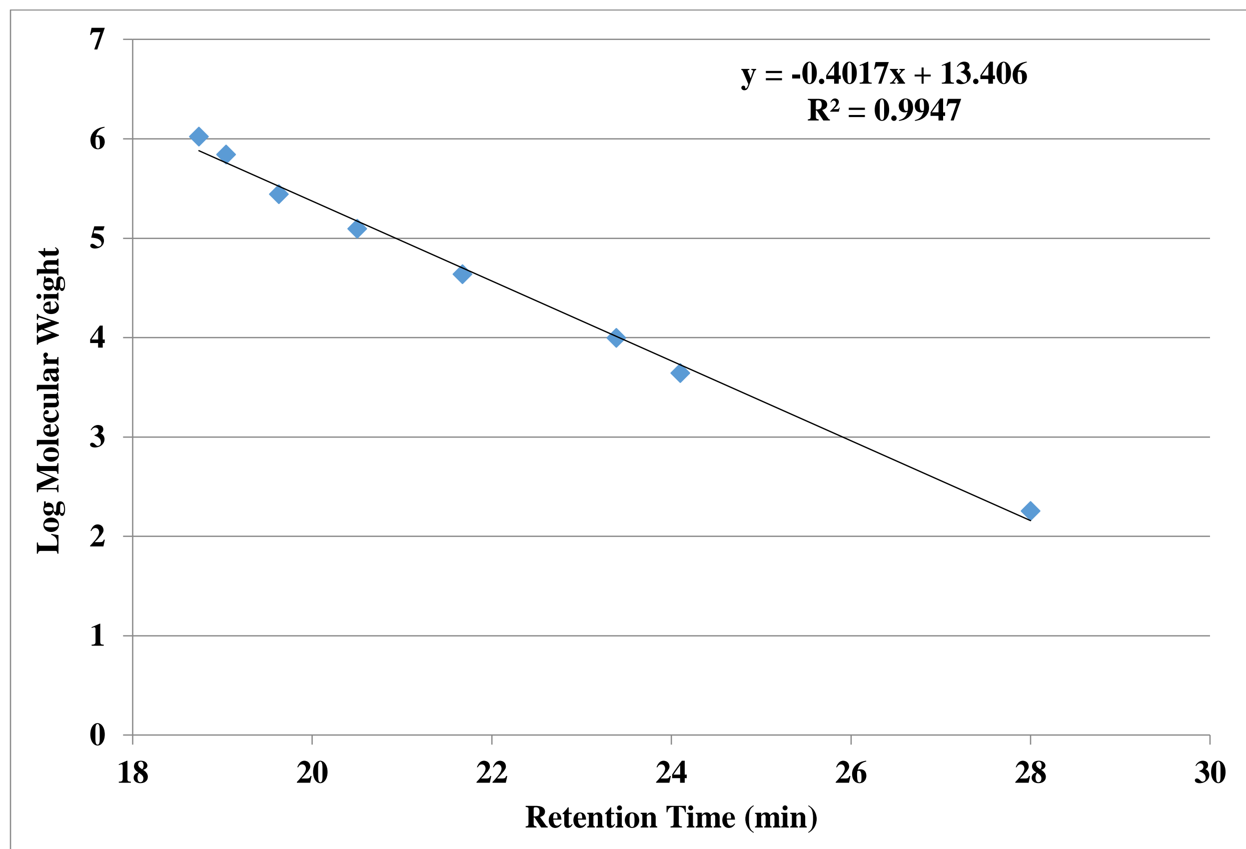

Figure 1a. Standard curve used to estimate the degree of polymerization of debranched starches without or with the β-amylase treatment.

Debranching of waxy maize starch resulted in linear starch chains with a dominant peak at retention time (RT) 24.4 min, which corresponded to a peak DP 25 (Figure 1b). Approximately 54% of starch chains in the debranched waxy maize starch were longer than DP 20, which could participate in complex formation with hexanoic acid.[29]

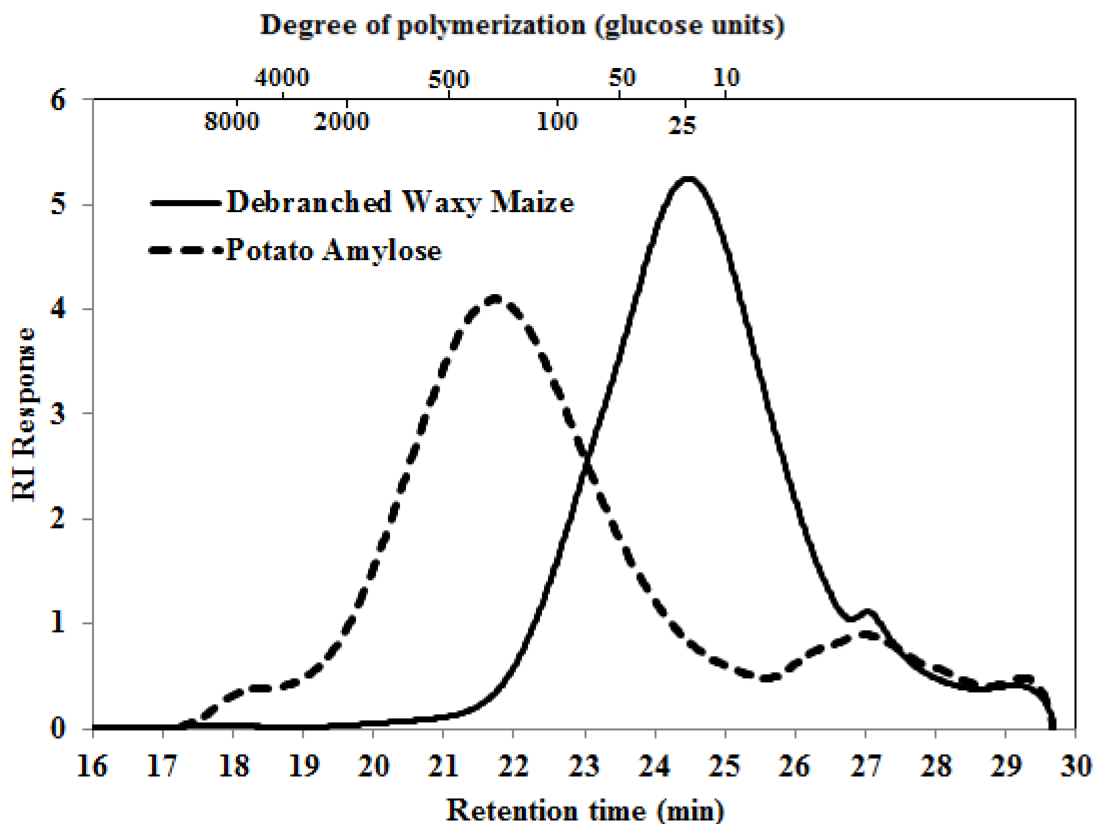

Figure 1b. Molecular size distribution of potato amylose and debranched waxy maize.

The peak DP 25 of debranched waxy maize starch obtained in this study was similar to Nakazawa and Wang[30] of a DP 27, but higher than previous studies with Cai and Shi[31] reporting a DP of 9 and Adkins et al.[32] reporting a DP of 17. Potato amylose showed a major peak at RT of 21.6 min, which corresponded to a peak DP ~330, and a shoulder at RT of 18.3 min, which corresponded to a peak DP ~7000. The peak DPs of debranched waxy maize and potato amylose, i.e. DP 25 and 300, were within the range of synthesized amylose (DP 9-1000) in a complexation microcalorimetric study by Wulff & Kubik.[22] They reported that linear starch chains with a DP 9 was sufficient to take part in complex formation. The amylose fraction with a peak DP 7000 in potato amylose in this study was presumably not involved in complexation because it exceeded

the reported starch chain length for fatty acid complexation according to Gelders et al.,[6] who found that amylose with a DP > 400 was not effective in forming stable complexes with fatty acids. Other studies reported that DP 18-24 glucose units was effective to complex with one fatty acid or monoacyl glycerol of 14-18 carbons,[33] DP 30-40 with two palmitic acid (C16:0)[5], DP 34 with two stearic acid,[7] and DP 40 with two docosanoic acid (C22:0).[6]

Iodine Affinity. Iodine affinity (IA) via potentiometric titration is traditionally used to measure the apparent amylose content in native starch based on complexation between amylose and iodine, although it is known that amylopectin long chains also interact with iodine to overestimate apparent amylose content. In this study, IA was used to relate to complexation with hexanoic acid. Debranching increased the IA of waxy maize starch from 0.2 to 2.9, which was equivalent to 1% and 15.4 % apparent amylose content, respectively (Table 1).

Table 1. Iodine Affinity and Apparent Amylose Content*[a]* for Native and Debranched Waxy Maize and Potato Amylose.

starch	iodine affinity	% apparent amylose content[b]
waxy maize starch		
native	0.2±0.0	1.0±0.1
debranched	2.9±0.7	15.5±3.9
potato amylose	13.9±0.1	70.0±0.5

[a]Apparent amylose content was determined by comparing against the typical iodine affinity value of purified linear fraction of the corn and potato amyloses, which are 19.0% and 19.9%, respectively.

The IA of potato amylose was 13.9, which was equivalent to ~70.0% apparent amylose content. The high apparent amylose content suggests that potato starch might interact more with hexanoic acid than debranched waxy maize starch because of the presence of more linear starch chains. When the apparent amylose content of starches (15.4 and 70 % for debranched waxy

maize starch and potato amylose, respectively) are compared with their respective molecular weight distribution (Figure 1b), the results indicate that the minimum DP of 70 may be preferred to complex with iodine. It is possible that the minimum DP required to complex with hexanoic acid is also approximately around 70 because Mikus et al.[34] previously reported that iodine and fatty acids occupied the same position in the starch helix.

Isothermal titration calorimetry. The thermodynamic parameters obtained for the interactions of debranched waxy maize starch and potato amylose with hexanoic acid of three concentrations at two temperatures are presented in Table 2, and the titration curves for the ITC measurements are displayed in Figures 2-3 for debranched waxy maize starch and in Figures 4-5 for potato amylose. The curves show that the titration of hexanoic acid triggered an exothermic co-operative binding event, and the binding between debranched waxy maize starch or potato amylose and hexanoic acid was stabilized by enthalpy of binding. The titration curve of debranched waxy maize starch with 20 mM hexanoic acid at 25 °C showed that the first few injections produced large exothermic peaks up to the 16th injection, and thereafter there was a decline in the peak height of the signals indicating, that starch chains were becoming saturated with hexanoic acid (Figure 2a). The transfer of the non-polar tails of hexanoic acid from buffer into starch helices to form starch-hexanoic acid complexes was responsible for the exothermic reaction observed.[24,26] This is in agreement with previous studies by Wulff & Kubik[22] and Wangsakan et al,[24,26] using maltodextrins with DE between 5 and 25, that when the non-polar part of surfactant molecules was transferred from a highly polar environment into the less polar environment of maltodextrin helix, an exothermic reaction was produced.

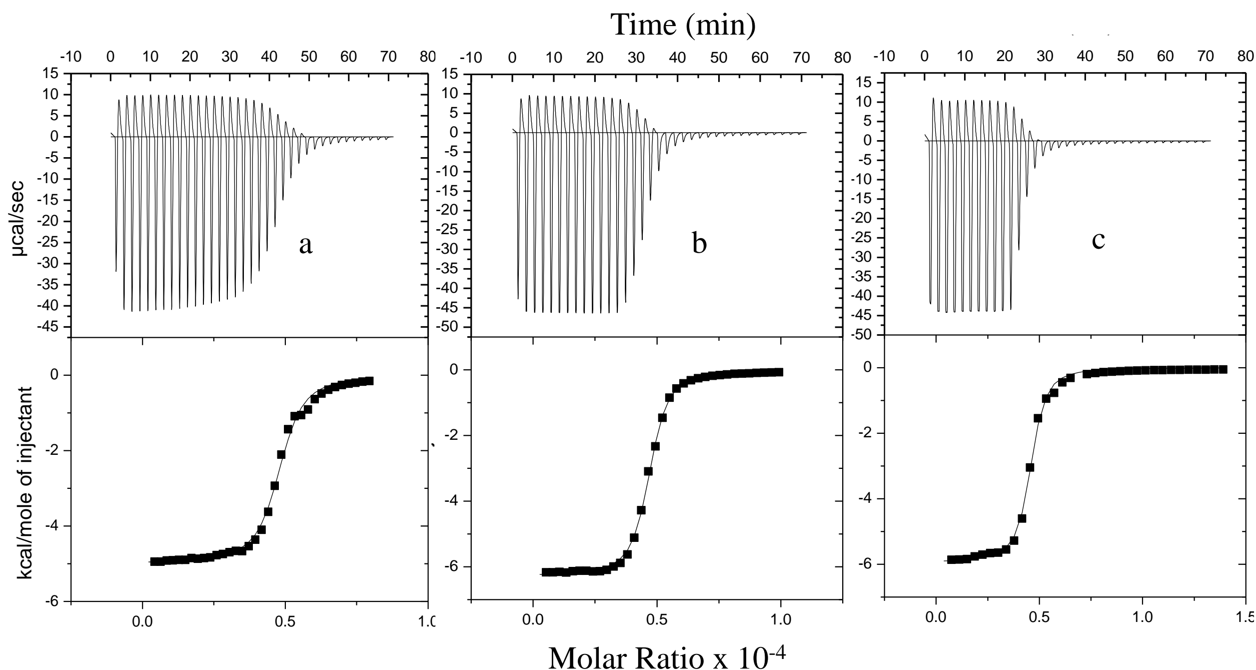

Figure 2. ITC profile of debranched waxy maize and hexanoic acid run at 25 °C (a) 20 mM (b) 25 mM (c) 35 mM.

When hexanoic acid concentration was increased to 25 mM and 35 mM an earlier decline in exothermic peak signal was observed during titration (Figure 2b and 2c). This indicates that as hexanoic acid concentration was increased, there was more hexanoic acid available to bind to starch per injection, consequently, the debranched waxy maize starch chains became saturated faster and the amount of heat produced during binding reduced. Eventually, the debranched waxy maize starch chains were all saturated with hexanoic acid, and only the heat of dilution from the titration was observed. A similar trend for reduction in heat signal was observed when hexanoic acid concentration increased for debranched waxy maize starch titrated at 45 °C (Figure 3) and also for potato amylose titrated at 25 and at 45 °C (Figures 4 and 5). It was noted that saturation of starch chain and reduction in heat signal occurred earlier for debranched waxy maize starch compared to potato amylose at both 25 and 45 °C. This suggests that complexation

between debranched waxy maize starch and hexanoic acid may be more favored than with potato amylose.

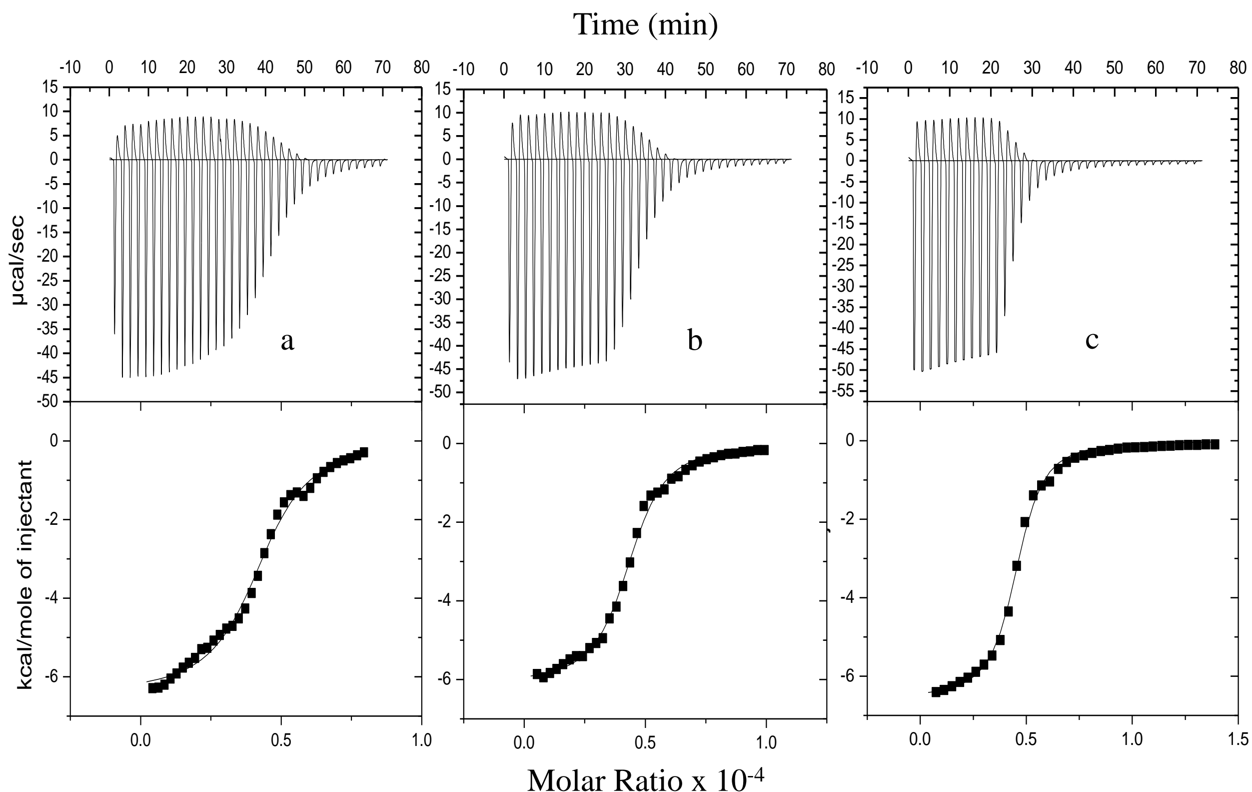

Figure 3. ITC profile of debranched waxy maize and hexanoic acid run at 45 °C (a) 20 mM (b) 25 mM (c) 35 mM .

Because starch is polydisperse and polymolecular in nature, there was a wide range of molecular weight distribution of starch chains in both debranched waxy maize and potato amylose. Thus the fitting of the data was more difficult and the variability of entropy values (ΔS) was high. The ΔS values may not represent the true condition of the binding of starch to hexanoic acid and therefore are not included.

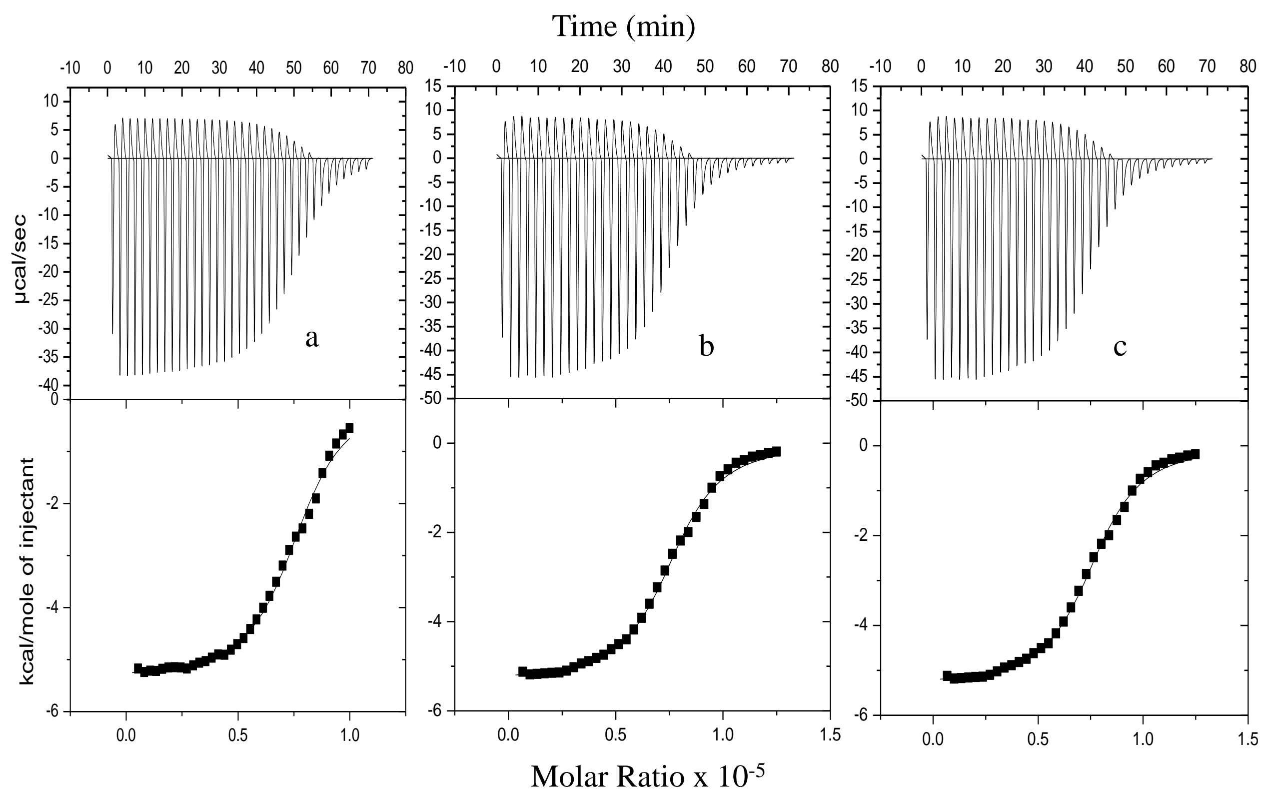

Figure 4. ITC profile of potato amylose and hexanoic acid run at 25 °C (a) 20 mM (b) 25 mM (c) 35 mM.

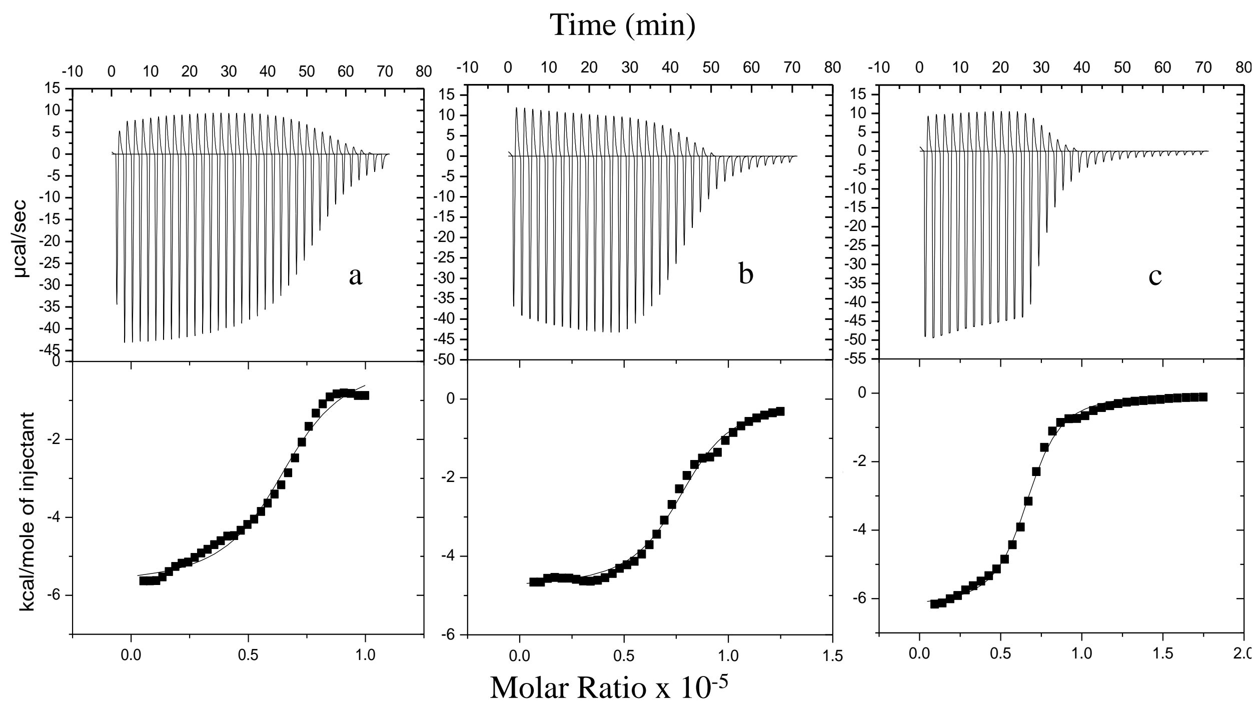

Figure 5. ITC profile of potato amylose and hexanoic acid run at 45 °C (a) 20 mM (b) 25 mM (c) 35 mM.

This in agreement with Mun et al.[27] that reported that the amount of surfactant (SDS) and dodecyl trimethylammonium bromide (DTAB) that will bind to cycloamylose chains with varying DP of 24 to 44 will differ because of the difference in starch chain length and how far the surfactants penetrate the cavity of the cycloamylose. They suggested that these factors will determine the entropy of each complexation reaction, which will differ from one another.

Table 2. Thermodynamic parameters of debranched waxy maize starch and potato amylose when titrated with varying concentrations of hexanoic acid at 25 and 45°C.[a]

		25 °C			45 °C		
starch	hexanoic acid (mM)	n $\times 10^{-4}$	Ka (M^{-1})	ΔH (kcal/mol)	n $\times 10^{-4}$	Ka (M^{-1})	ΔH (kcal/mol)
Debranched waxy maize	20	0.42±0.01	6.24±2.35	-6390±6	0.43±0.04	1.98±0.17	-5971±152
	25	0.42±0.04	4.02±0.60	-6125±518	0.41±0.01	1.31±0.21	-5638±399
	35	0.35±0.01	2.82±0.28	-6694±29	0.39±0.05	1.69±0.75	-5043±76
Potato Amylose	20	0.07±0.01	0.51±0.33	-6296±41	0.08±0.00	0.12±0.04	-4073±680
	25	0.07±0.01	0.40±0.25	-6293±57	0.07±0.00	0.16±0.07	-5894±267
	35	0.06±0.00	0.34±0.20	-6250±346	0.07±0.00	0.18±0.08	-5849±421

[a]Data of at least two measurements with standard deviation. Means in a column not sharing the same letter are significantly different based on Tukey's honestly significant difference test ($p < 0.05$).

The enthalpies of binding (ΔH) representing the true value of the reaction at various concentrations of hexanoic acid were all negative (Table 2), indicating a spontaneous and favorable binding reaction of mostly hydrophobic in nature. There was no particular trend in entropy values for the binding of hexanoic acid to debranched waxy maize starch and potato amylose at 25 °C, and for all hexanoic acid concentrations used, the entropy of binding for 20 and 35 mM hexanoic acid to debranched waxy maize starch at 45 °C was positive, and it increased with increasing hexanoic acid concentration. The entropy for the binding of potato amylose to hexanoic acid at 45 °C was also positive, but it decreased with increasing hexanoic acid concentration. These entropy results indicate that at 45 °C as the hexanoic acid concentration increased, the degree of order of the system increased for the debranched waxy maize starch, suggesting a favorable reaction, but the opposite trend was observed for potato amylose.

The binding affinity (K_a) as observed from the titration curves showed that K_a for debranched waxy maize starch and potato amylose decreased from 25 to 45 ºC, except for potato amylose binding to 35 mM hexanoic acid. The similar K_a values at 35 mM hexanoic acid at both 25 and 45 ºC indicate that the increase in hexanoic acid concentration might play a more important role in complex binding for potato amylose. In addition, the K_a values of debranched waxy maize starch were significantly higher than those of potato amylose at the same hexanoic acid concentration and temperature, supporting earlier results that there was a higher binding affinity of hexanoic acid to debranched waxy maize starch. The high standard deviation of K_a values was assumed to be similar to that of the ΔS values explained earlier.

There was no noticeable change observed in the (n) values for the binding occurring at 25 and 45ºC and with increasing concentration of hexanoic acid for debranched waxy maize starch

and potato amylose at ~0.42 and ~0.06, respectively (Table 2). These values indicate that on average for every molecule of hexanoic acid bound, approximately 2.5 molecules of debranched waxy maize starch and approximately 15.8 molecules of potato amylose was involved in the reaction. These results agree with the HPSEC results that debranched waxy maize starch had a lower peak DP 25 compared with potato amylose of a peak DP 330, therefore more starch chains were available for binding for debranched waxy maize starch than for potato amylose.

In addition to the challenge of polymolecular nature of starch, another challenge in the present study was the possibility that two opposing events can be observed from all the titration curves, with the first signifying the occurrence of an exothermic reaction and a second reaction occurring in the endothermic region which may signify the heats of dilution (Figure 2-5). The exothermic reaction was the interaction of starch chains and hexanoic acid, which however triggered a second reaction that was endothermic in nature. It is important to note that the second endothermic reaction occurred only after the injection of hexanoic acid and was proposed to be the heats of dilution obtained from the reassociation of starch chains, or a conformational change occurring in the structure of the starch chains, which might have changed the sequence of the binding reaction. Therefore the heat of reaction, and other thermodynamic parameters estimated cannot be fully attributed to the binding between starch chains and hexanoic acid alone, but may also include other conformational changes occurring with the starch chains.

CONCLUSIONS

Nevertheless, the ITC results demonstrate that hexanoic acid bound faster to debranched waxy maize starch than to potato amylose, implying that the shorter average DP 25 of debranched waxy maize starch, became saturated faster when binding to hexanoic acid than potato amylose of a higher average DP 330. The ITC observations are not consistent with the IA

values, which showed more iodine binding with potato amylose than with debranched waxy maize starch. The longer time it took for potato amylose to become saturated with hexanoic acid may be due to the long chain length of potato amylose to interact properly with hexanoic acid to form stable complexes. The structural differences between iodine and hexanoic acid may also contribute to their differences in binding. The present study showed that the binding of hexanoic acid to debranched waxy maize starch occurred much faster than to that of potato amylose due to shorter chain length of debranched waxy maize starch. The effect of hexanoic acid concentration had a greater impact on the K_a values than on the n values of the complexation. The present results suggest that shorter starch chains such as from the debranched waxy maize starch may be favored to achieve a faster complexation, and consequently increase the overall efficiency of complex formation.

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APPENDIX

Authorship Statement for Chapter 2

I verify that Emily Arijaje is the first author and completed over 51% of the research work published in the following article that is included in her dissertation: Complexation between Hexanoic Acid and Linear Starch Molecules using Isothermal Titration Calorimetry - Chapter 2

Dr. Ya-Jane Wang

V. CHAPTER 3: Effects of Enzymatic Modifications on Starch-Stearic Acid Complex Formation.

ABSTRACT

Debranched unacetylated and acetylated potato starches with two degrees of substitution 0.041 (low) and 0.078 (high) combined with or without β-amylase hydrolysis were prepared to form soluble and insoluble starch complexes with stearic acid. The effects of modifications on the complexation, thermal properties and X-ray patterns of soluble and insoluble complexes were investigated. Acetylation decreased the recovery of insoluble complexes but increased that of soluble complexes. Low acetylated, β-amylase-treated starch had a significantly increased amount of complexed stearic acid (123.1 mg/g) for insoluble complexes; high acetylated, β-amylase-treated starch had the highest complexed stearic acid (61.2 mg/g) for the soluble complexes. The melting temperature of the complexes decreased with acetylation. All β-amylase treated acetylated complexes displayed the V-type diffraction pattern with peaks at $2\theta = 7.4°$, 12.9° and 20°. These results suggest that starch can be modified by acetylation, debranching, and/or β-amylase to produce significant quantities of soluble starch-stearic acid complexes.

KEYWORDS: starch acetylation, starch-fatty acid complex, soluble complex, insoluble complex, X-ray diffraction pattern

INTRODUCTION

Amylose is a component of starch and essentially linear in structure with repeating glucose units linked by α-D-(1→4) glucosidic linkages. Amylose adopts a helical structure and forms inclusion complexes through hydrophobic interactions with guest molecules, such as iodine,[1] alcohols,[2] fatty acids,[3-5] flavors[6,7] and genistein.[8]

Amylose-fatty acid complexes were first reported by Schoch and Williams[9] and have since been extensively studied. The characteristics of amylose-fatty acid complex are influenced by the starting materials used for the complexation reaction,[10] such as amylose from starch, purified amylose or synthesized amylose. Complexing with purified or synthesized amylose tends to yield more monodisperse complexes than with starch.[10] The stability, yield and organization of amylose-fatty acid complexes were found to be increased with an increase in fatty acid chain length[11] and saturation,[12] amylose chain length up to degree of polymerization (DP) of 400 for monodisperse amylose,[13] incubation temperature,[14] incubation time, and pH.[15] Nevertheless, the complexation yield between amylose and fatty acid is relatively low, ranging from ~3.5% for conjugated linoleic acid[5] and 6.9% for a mixture of linoleic and stearic acid.[15] The low yield is partly attributed to the stronger tendency for self-association of amylose than for complexation with other molecules.

Upon complexation, amylose and the included guest molecule form ordered crystalline structures, which become insoluble and precipitate out of solution. Most studies on amylose-fatty acid complexes have focused mainly on the preparation and characterization of these insoluble complexes. Upon complexation, the amylose-included molecule can be stabilized and protected from oxidation and light by amylose, thus exhibiting extended stability. Nevertheless, increase in crystallinity from complexation results in reduced solubility and increased resistance to enzyme

hydrolysis for the included molecules, which could ultimately limit its bioavailability. Therefore preparation of soluble complexes is of great importance to improve solubility and bioavailability of bioactive compounds for the prevention and treatment of diseases.

Chemical modification by cross-linking amylose with cyanide chloride has been used by Kubik and Wulff[16] to create a tailored amylose cavity that was ligand specific. Substitution is also commonly applied to starch to modify starch properties to increase solubility and reduce retrogradation.[17] When hydroxypropylated amylose at a degree of substitution (DS) of 0.075 was used to complex 4-tert-butylphenol (t-BP) and sodium dodecyl sulfate (SDS), there was an improvement in the water solubility of the complexes formed, and the modification of amylose had minimal impacts on its complexing ability.[18] Wulff et al.[19] reported that acetylated potato amylose derivatives at DS of 0.16 yielded soluble complexes with fenchone, a low molecular-weight flavor compound.

In the present study, native and acetylated potato starches were debranched and combined with or without an additional β-amylase treatment to prepare complexes with stearic acid (C18:0). Debranching of starch increased the amount of chains available for complexation by converting highly branched amylopectin into all linear chains. The β-amylase treatment shortened the chains by sequentially removing maltoses from the nonreducing end, which could lead to chains with more favorable chain lengths and, consequently, better complexing ability. Besides reducing retrogradation and increasing solubility of starch, acetylation could also improve complexation with stearic acid by enhancing the hydrophobicity of the starch helical cavity. The objectives of this study were to determine the effects of these treatments on the formation of soluble and insoluble complexes and their complexation yields and physicochemical properties.

MATERIALS AND METHODS

Materials. Potato starch was obtained from Penford Food Ingredients (Centennial, CO, USA) and used without further treatment. Isoamylase (specific activity 59,000 units/mg protein) was purchased from Hayashibara Biochemical Laboratories Inc. (Okayama, Japan). β-amylase from *Bacillus cereus* (specific activity=2484 units/mg protein) was purchased from Megazyme International Ireland Ltd. (Wicklow, Ireland). Stearic acid was from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of ACS grade.

Acetylation of Starch. Acetylation was carried out following the method described by Wang and Wang.[20] Starch 160 g dry basis (db) was weighed into a 2-L reaction vessel, and water was added to make up a final weight of 457.2 g. The mixture was stirred and hydrated for 30 min, and then the pH was adjusted to 8-8.5 with 1 M NaOH. Acetic anhydride (6.4 g, 4% based on starch db) was used for a low acetylation, or 9.6 g (8% based on starch db) was used for a high acetylation. Acetic anhydride was added very slowly to the starch mixture while the starch slurry was maintained pH between 8-8.5 and stirred. After the addition of acetic anhydride, the slurry was left to stand for 60 min with stirring. The resulting mixture was adjusted with 1 M HCl to a pH of 5.5, washed with a 3-fold volume of deionized water, filtered, and dried in an oven at 40 °C for 24 h. The acetyl content of starches was determined by the method of McComb and McCready[21] and expressed as degree of substitution (DS).[22]

Enzymatic Modification of Starch.

Debranching. Starch (15 g wet basis) was added to 400 mL of water (3.75% w/v) and placed in a boiling water bath for 1 h with constant stirring to achieve complete gelatinization. The solution was equilibrated in a water bath at 45 °C, adjusted to pH 3.5 with 0.5 M HCl, and added with 0.5% (v/w starch db) isoamylase and then incubated at 45 °C with constant stirring for 48 h.

The starch was recovered with four-fold volume of pure ethanol, centrifuged, dried at 40 °C for 48 h and milled using a UDY cyclone mill (UDY Corp., Ft. Collins, CO, USA) fitted with a 0.5-mm screen.

β-Amylase Treatment. A portion of the debranched starch was further subjected to β-amylase hydrolysis to reduce the DP. After incubation with isoamylase for 48 h, the starch slurry was adjusted to pH 6.5 with 0.5 M NaOH, and incubated with 0.5% (v/w starch db) β-amylase at 40 °C for 4 h. The enzyme reaction was terminated by boiling for 15 min. Starch was recovered by precipitating with 4-fold volume of pure ethanol, dried at 40 °C for 48 h and milled using the UDY cyclone mill fitted with a 0.5-mm screen.

Characterization of Starch Structure. The molecular-size distribution of debranched unacetylated and acetylated starch with or without β-amylase treatment prior to complexation was characterized using a high-performance size-exclusion chromatography (HPSEC) system (Waters Corp., Milford, MA, USA). Starch (10 mg) was dissolved in 5 mL of 90% dimethyl sulfoxide (DMSO), boiled for 1 h, and filtered through a 5.0-μm filter prior to injection into the HPSEC system. The HPSEC system consisted of a guard column (OHpak SB-G, 6.0 × 500 (mm) i.d. × length), two Shodex columns (OHpak KB-804 and KB-802, both 8.0 × 300 (mm) i.d. × length), a 200 μL injector valve (model 7725i, Rheodyne, Cotati, CA, USA), an inline degasser, a model 515 HPLC pump, and a model 2414 refractive index detector. The mobile phase of 0.1 M ammonium acetate with 0.02% sodium azide was eluted at a flow rate of 0.4 mL/min. The temperature of the columns was maintained at 55 ºC and that of the detector at 40 ºC. Dextran standards with molecular weights of 5,200, 11,600, 23,800, 48,600, 273,000 and 410,000 g/mole from Waters Corp. (Milford, MA, USA) and 1,050,000 g/mol from Sigma-Aldrich (St. Louis, MO) were used to establish the calibration curve. An Ultrahydrogel 250 column (Waters Corp.,

Milford, MA, USA) was used for analyzing the molecular size distribution of starches recovered after complexation with stearic acid.

Amylopectin chain-length distribution was characterized by high-performance anion-exchange chromatography equipped with pulsed amperometric detection (HPAEC-PAD) according to the method of Wong and Jane.[23] The HPAEC-PAD system (Dionex ICS-3000, Sunnyvale, CA, USA) consisted of the components of AS40 autosampler, a single pump, detector/chromatography module (DC), a 4 × 50 mm CarboPac PA1 guard column, and a 4 × 250 mm CarboPac PA1 analytical column. Starch (20 mg) was boiled in 3.2 mL of distilled water for 1 h, cooled, and then filtered through a 0.45 μm filter (NYL w/GMF, Whatman, Clifton, NJ, USA) prior to injection into the HPAEC-PAD system. The mobile phase consisted of two eluents, A (150 mM NaOH) and B (150 mM NaOH containing 500 mM sodium nitrate) and was eluted at a flow rate of 1 mL/min. The gradient program was as follows: 94% of eluent A at 0 min, 92% at 11 min, 87% at 31 min, 80% at 81 min, 75% at 105 min and 94% at 106 min. Column temperature was at 25 °C, and injection volume was 25 μL. Sugar standards, including glucose (DP1), maltose (DP2), maltotriose (DP3), maltotetraose (DP4), maltopentoase (DP5), maltohexaose (DP6), and maltoheptaose (DP7), were used as calibration standards to identify the chromatographic peaks. Each successive peak after DP 7 was considered to represent one glucose unit longer than the previous peak. The chains were divided into DP ranges and classified as follows: A chains (DP 6-12), B1 chains (DP 13-24), B2 chains (DP 25-36), and B3+ chains (DP 37+).[24] The average chain length was calculated as the cumulative sum of the product of DP and percentage relative areas for all the identified peaks.

Complexation of Starch and Stearic Acid. The starch solution (3.75% w/v) after debranching and with or without the β-amylase treatment as previously described was adjusted to pH 7.0 prior

to the addition of 1 g of stearic acid. The mixture was heated at 80 °C for 30 min with continuous stirring, and then the temperature was lowered and maintained at 45 °C overnight with continuous stirring. Then the starch-stearic acid mixture was centrifuged at 7000*g* for 10 min, from which the precipitate, that is "*insoluble complex*", was obtained, and the "*soluble complex"* was recovered from the supernatant by precipitation with a 4-fold volume of pure ethanol. Uncomplexed stearic acid was removed from both the *insoluble* and *soluble* complexes by rinsing with 95% ethanol using a labquake shaker rotisserie (Barnstead/Thermolyne, Dubuque, IA, USA) at room temperature for 2 h. Complexes were recovered by centrifugation at 7000*g* for 10 min, dried at 40 °C for 48 h, milled using a mortar and pestle, sieved through a 250 mm sieve, and stored for further analysis.

Hydrolysis of Complexes and Stearic Acid Analysis. To the complex (100 mg), both soluble and insoluble, was added 10 mL of 1 M HCl and heated in a boiling water bath for 1 h. The complex mixture was cooled, and 5 mL of hexane was added to extract the released stearic acid by rotation on the rotary shaker for 1 h. The hexane layer was recovered, and the previous step was repeated with another 5 mL hexane for 1 h. Boron trifluoride-methanol was added to the recovered hexane phase to prepare fatty acid methyl esters, and an internal standard of methyl heptadecanoate (~ 1 mg) was subsequently added. The fatty acid methyl esters extracted in the hexane layer were injected into a gas chromatography (GC) system (GC-2010, Shimadzu, Kyoto, Japan) equipped with a BP 21 capillary column (30 m × 0.25 mm i.d.; SGE Inc., Austin, TX, USA) with a flame ionization detector (FID), and responses were collected by a Shimadzu GCsolution Workstation 2.3 (Kyoto, Japan). The temperature of the column oven was equilibrated at 130 °C for 2 min, ramped at 10 °C /min to 250 °C, and maintained at 250 °C for 3 min. The injector and detector temperatures were set at 250 and 270 °C, respectively. Stearic

acid was methylated and the concentrations of stearic acid were determined from a standard curve prepared by using stearic acid solution (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL) containing the internal standard of methyl heptadecanoate (0.5 mg/mL).

Physicochemical Properties. The thermal stability of all complexes was measured using a differential scanning calorimeter (DSC, Pyris-Diamond, PerkinElmer, Shelton, CT, USA). Starch samples (approximately 8 mg) were weighed into stainless steel pans, 16 μL of distilled water was added with the aid of a microsyringe, and the pans were hermetically sealed. The pans were equilibrated for at least 1 h at room temperature before scanning. The samples were scanned from 25 °C to 180 °C at a rate of 10 °C/min. The onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c), and enthalpy (ΔH) of the endotherms were calculated using the Pyris data analysis software.

X-ray diffraction pattern. The powder X-ray diffraction pattern of complexes was determined using a diffractometer, (PW1830 MPD, Philips, Almelo, The Netherlands). The generator voltage was set at 45 kV and the current at 40 mA. The sample was scanned over the 2θ angular range from 5° to 35° with a step size of 0.02° and time of 1 s per step.

Statistical Analysis. All measurements were conducted in replication, and the data were analyzed using JMP software (SAS Institute Inc., Cary, NC, USA) and the means were compared using Tukey's honestly significant differences (HSD) test.

RESULTS AND DISCUSSION

Degree of Substitution. The DS values of the prepared low acetylated and high acetylated starches were 0.041 and 0.078, respectively. After both acetylated starches were debranched and treated with or without β-amylase and complexed with stearic acid, the insoluble and soluble fractions were recovered and analyzed for their structures and properties. It was found that the

soluble fractions had significantly higher DS of acetylation, 82-173% higher, than their insoluble counterparts for the same treatment (Table 1). The differences in DS between the soluble and insoluble fractions of acetylated starch complexes were apparently responsible for the type of complexes formed because starch solubility increases with an increase in acetyl groups.

Table 1. Degree Of Substitution (DS) of Acetylated Starches and Stearic Acid Complexes[a].

starch	β-amylase treatment	type of complex	DS
Low acetylated starch	N/A	N/A[b]	0.041±0.001f
Low acetylated starch complex	No	Soluble	0.063±0.002d
		Insoluble	0.029±0.000g
	Yes	Soluble	0.093±0.002b
		Insoluble	0.034±0.000f
High acetylated starch	N/A	N/A	0.078±0.001c
High acetylated starch complex	No	Soluble	0.089±0.002b
		Insoluble	0.049±0.001e
	Yes	Soluble	0.133±0.002a
		Insoluble	0.052±0.002e

[a]At least replicate samples were prepared for each complex. Data of two measurements with standard deviation. Means in a column not sharing the same superscript letter are significantly different based on Tukey's honestly significant difference test ($p < 0.05$).
[b]N/A- Not Applicable.

A further increase in DS was observed when acetylation was combined with β-amylase treatment for both soluble and insoluble fractions. Because starch was acetylated in its native state, most acetyl groups were present in the amorphous lamella close to the branching points. Therefore,

most acetyl groups were retained after β-amylase hydrolysis, resulting in an increase in DS of acetylation for samples treated with β-amylase.

Wulff et al.[19] reported that for the formation of soluble complexes of fenchone, a minimum DS of 0.16 was required for acetylated amylose, and only insoluble complexes were formed when acetylated amylose with a DS < 0.16 was used. In the present study, soluble complexes were produced from acetylated potato starch with DS ≥ 0.063. The difference in the DS observed in the two studies may be because starch was used in this study, whereas amylose was used by Wulff et al.[19] Although a different guest molecule was used in the present study, these results suggest that amylopectin chains participated in complexation, and the required DS of acetylation for soluble complex formation was proportional to the DP of amylose and amylopectin chains. Nevertheless, the present results are in agreement with Wulff et al.[19], that there is a minimum DS of acetylation for the formation of soluble complex.

Complex Recovery and Complexation Yield. The recovery of complex was calculated by comparing the weight of recovered fractions to the weight of initial materials. The highest recovery for the insoluble complex was 0.72 g/g from the unacetylated starches with the β-amylase treatment. The recovery of unacetylated insoluble complex is in agreement with previous studies,[5,25] in which only insoluble fractions were reported. The total recovery, including both soluble and insoluble complexes, was highest for the unacetylated starch and decreased with increasing acetylation, which was probably because the increased solubility of starches from acetylation resulted in a significant reduction complex recovery (Table 2). These results demonstrate that the presence of acetyl groups the increased solubility of starch and prevented the precipitation of starch-stearic acid complexes after complexation, thereby resulting in a lower recovery for insoluble complexes but a higher recovery for soluble complexes.

Table 2. Complex Recovery and Percentage Stearic Acid Content Recovered from All Complexes[a].

starch	type of complex	β-amylase treatment	recovery (g/g)	stearic acid in complex (mg/g)
unacetylated	Soluble	no	0.24±0.00d	15.7±1.5f
	Insoluble	no	0.58±0.00a	42.7±5.6d
	Soluble	yes	0.11±0.01e	33.8±5.4d,e
	Insoluble	yes	0.72±0.04a	63.1±1.2c
low acetylated	Soluble	no	0.26±0.00c,d	23.4±1.3e,f
	Insoluble	no	0.32±0.01c,d	101.0±5.9b
	Soluble	yes	0.28±0.01c,d	34.6±2.9d,e
	Insoluble	yes	0.47±0.01b	123.1±4.2a
high acetylated	Soluble	no	0.26±0.02c,d	36.8±7.4d
	Insoluble	no	0.28±0.02c,d	40.3±3.8d
	Soluble	yes	0.38±0.02b,c	61.2±12.2c
	Insoluble	yes	0.36±0.02c	16.6±1.9f

[a] Data of at least two measurements with standard deviation. Means in a column not sharing the same superscript letter are significantly different based on Tukey's honestly significant difference test ($p < 0.05$).

The additional β-amylase treatment increased recovery for the insoluble fractions of acetylated and unacetylated starches and for the soluble fractions of high acetylated starch. When acetylated starches received the β-amylase treatment, there was presumably an increase in chain lengths of starch chains that would favor for the formation of both insoluble and soluble complexes, thus resulting in an increase in complexation. However, for β-amylase-treated unacetylated starches the recovery of the insoluble complex increased, which was probably due to the absence of acetyl groups. The absence of acetyl groups may have led to an increase in

starch reassociation and a reduction in solubility of unacetylated starches, which ultimately prevented complexes from staying in solution. The recovery of soluble fractions from acetylated-only starch was similar to that from the unacetylated starch at 0.26 g/g, implying that acetylation alone might not be sufficient to increase the recovery of soluble complexes. The recovery of soluble fractions of acetylated starches increased when the β-amylase treatment was incorporated, and this increase became more noticeable with the high acetylated starch. The increase in recovery of the soluble fraction of high acetylated was ascribed to the increase in the acetyl groups that promoted the preference of stearic acid to interact with the hydrophobic cavity of starch helix. This may be ascribed to the orientation of acetyl groups toward the cavity, thereby increasing overall hydrophobicity of the cavity and encouraging more stearic acid interaction, and this increased complexation.[26]

Stearic acid was recovered from all starch complexes, and the amount of stearic acid recovered was higher in the insoluble fractions than in the soluble fractions, except for the high acetylated and β-amylase treated starch (Table 2). The highest amounts of stearic acid were recovered from the insoluble fractions of low acetylated starches fractions without (101.0 mg/g) and with (123.1 mg/g) the β-amylase treatment. Lui et al.[27] reported that acetylated pea starch with DS of 0.1 had a reduced iodine affinity of ~4.4% compared to native pea starch with a value of ~6.4%. They concluded that acetyl groups interfered with complex formation and reduced complexation yield. Similarly, Wulff et al.[19] reported that the complexing ability of amylose with fenchone decreased when amylose was modified with hydroxypropyl, hydroxyethyl, acetyl, and carboxymethyl groups. In contrast, the present results show that although acetylation of starch decreased the recovery of the insoluble fractions, the amount of complexed stearic acid was

increased by 95-135% at a low acetylation level with or without the β-amylase treatment, but then decreased at a high acetylation level.

For the soluble complexes, both acetylation and β-amylase treatment enhanced the complexation of stearic acid. The high acetylated, β-amylase-treated starch had the highest amount of complexed stearic acid (61.2 mg/g), which was about 81% higher than the amount of stearic acid recovered from the soluble fraction of unacetylated starch (33.8 mg/g). The present results demonstrate that there is an optimum DS of acetylation to increase the amount of complexed stearic acid with starch in both soluble and insoluble complexes. This supports our hypothesis that apart from acetylation, an optimum starch chain length may also be critical for increasing the complexation yield of stearic acid and formation of soluble complexes.

Molecular Size Distribution. The standard curves used to estimate the degree of polymerization of debranched starches without or with the β-amylase treatment and that of their complexes are displayed in Figure 1a and b, respectively. The molecular size distributions of the debranched unacetylated and acetylated starches prior to complexation with stearic acid are also displayed in Figure 1c. For the unacetylated starches, the chromatogram shifted to lower DP with the β-amylase treatment, and the proportion of amylose fraction between 26-31 min was clearly reduced. The hydrolysis by β-amylase reduced the molecular size of amyloses to the range of amylopectin long chains and also lowered the molecular size of amylopectin chains. For the acetylated starches, the proportion of the amylose fraction increased with acetylation, and a further increase was noted with the additional β-amylase treatment. This increase was attributed to an increase in the hydrodynamic volume of the starch due to the presence of acetyl groups rather than an increase in the proportion of amylose. This supports the DS results (Table 1) that

during β-amylase hydrolysis of acetylated starch most acetyl groups were retained and responsible for the increased hydrodynamic volume of the acetylated starches.

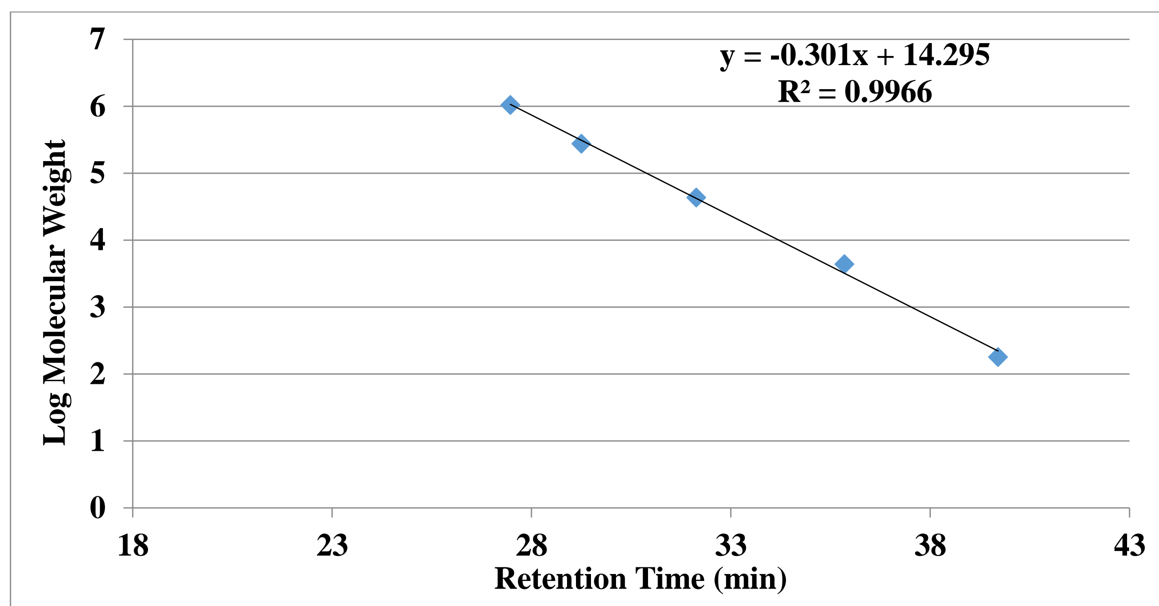

Figure 1a. Standard curve used to estimate the degree of polymerization of debranched starches without or with the β-amylase treatment.

The molecular size distributions of recovered soluble and insoluble fractions of all starches are presented in Figure 2. All starches displayed a peak at ~14.5 min, signifying the presence of amylose fractions. The peak at 14.5 min (~DP 3400) was more pronounced in the insoluble fractions than in the soluble fractions, became larger with acetylation, and then further increased when combined with the β-amylase treatment. These results support previous findings (Figure 1c) that the increase in amylose fraction was due to the presence of acetyl groups not hydrolyzed by β-amylase.

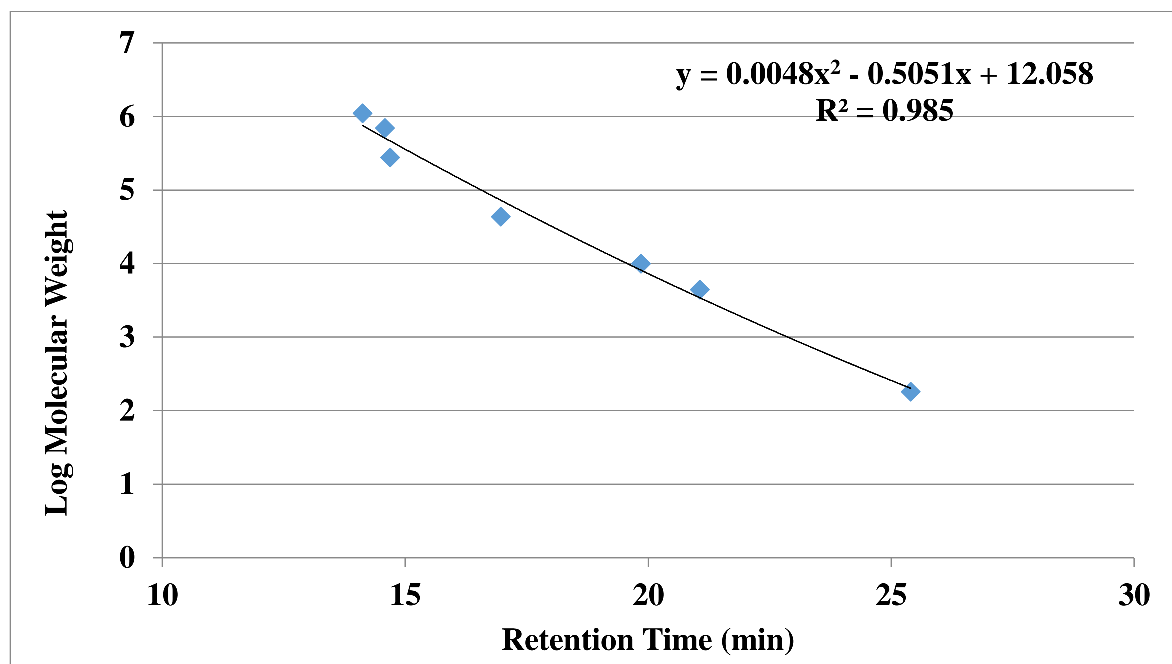

Figure 1b. Standard curve used to estimate the degree of polymerization of debranched starch complexes without or with the β-amylase treatment.

The acetylated fractions not treated with β-amylase also displayed two additional peaks at ~19.5 min of DP 67 and ~21 min of DP 23, which represent amylopectin long and short chains, respectively. However, the acetylated and β-amylase-treated starch fractions only had an additional peak around 19 min (DP 96).

It has been reported that approximately DP 18-24 glucose units are required for complexation of one fatty acid or monoacyl glycerol of 14-18 carbons,[10] DP 30-40 for two palmitic acid (C16:0),[28] DP 34 for two stearic acid,[29] and DP 40 for two docosanoic acid (C22:0).[13] Therefore it can be deduced that the minimum chains required for complexation of one or two stearic acid would be DP 18-40, and were available in both soluble and insoluble fractions in the present study.

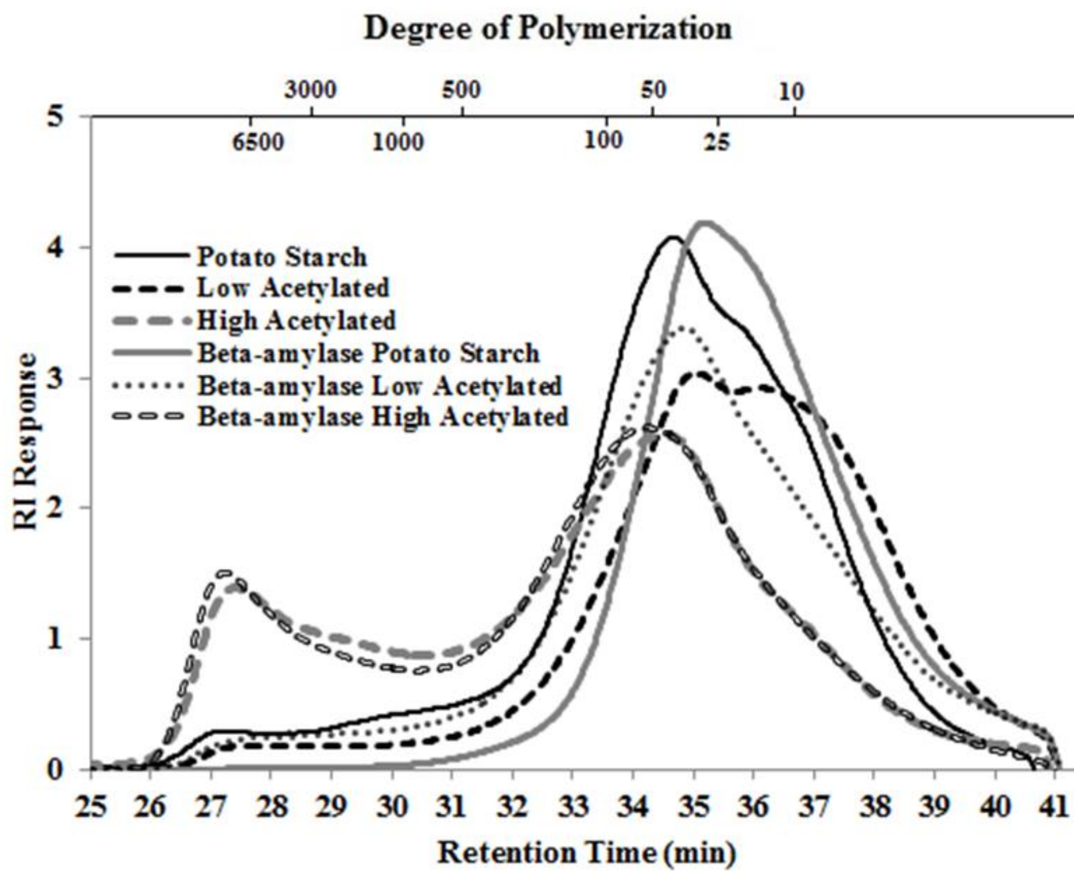

Figure 1c. Normalized HPSEC profiles of debranched only or debranched and β-amylase-treated unacetylated and low and high acetylated potato starch.

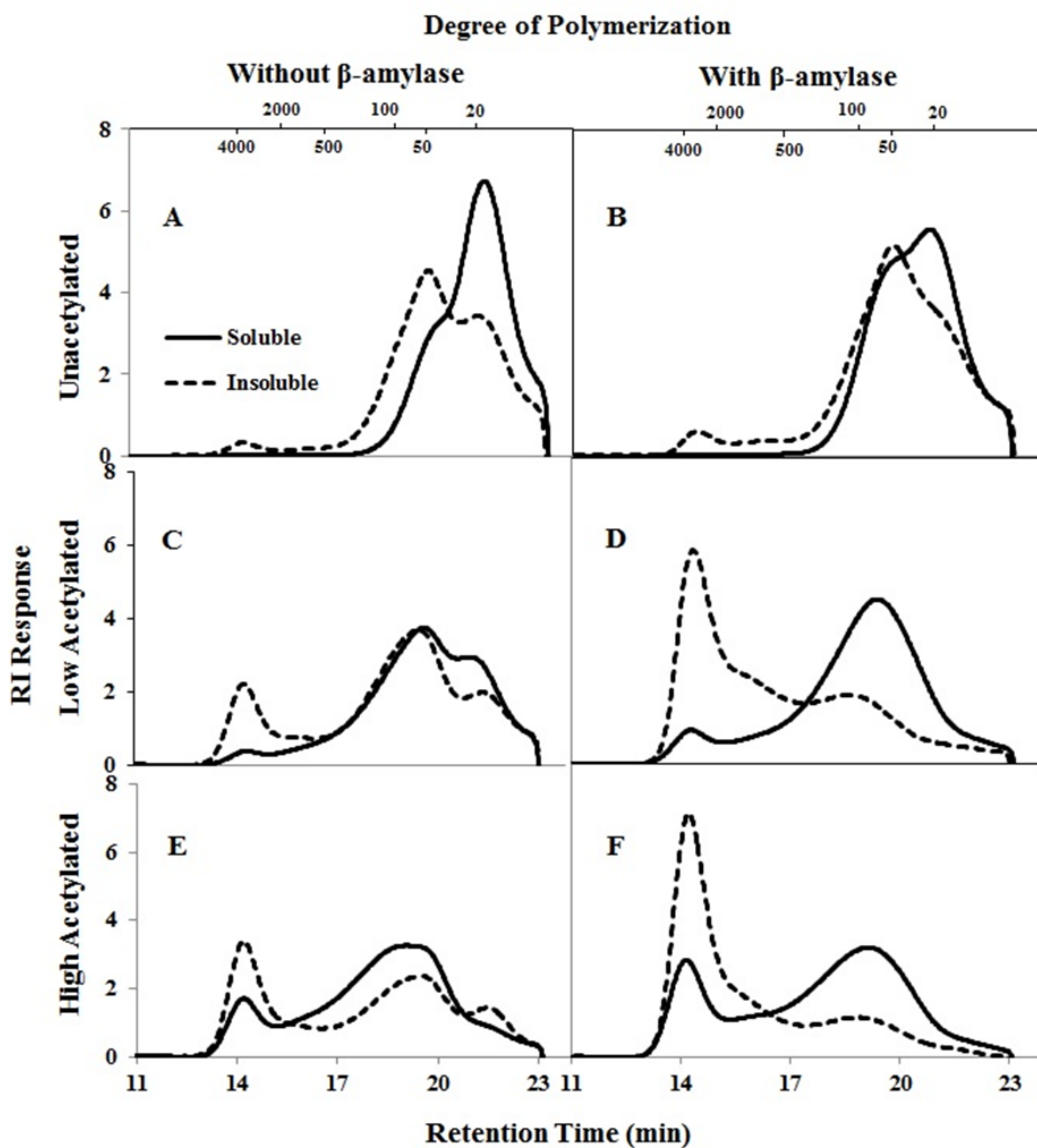

Figure 2. Normalized HPSEC profiles of recovered unacetylated and acetylated potato starches from the soluble and insoluble fractions after complexation.

The chain-length distributions of recovered starch chains from the soluble and insoluble fractions without and with β-amylase treatment are presented in Figures 3 and 4, respectively. The soluble fraction displayed a more unimodal-like distribution after the β-amylase treatment (Figure 4A, C, E) with peak DP 30 and 33 for low and high acetylated starches, respectively.

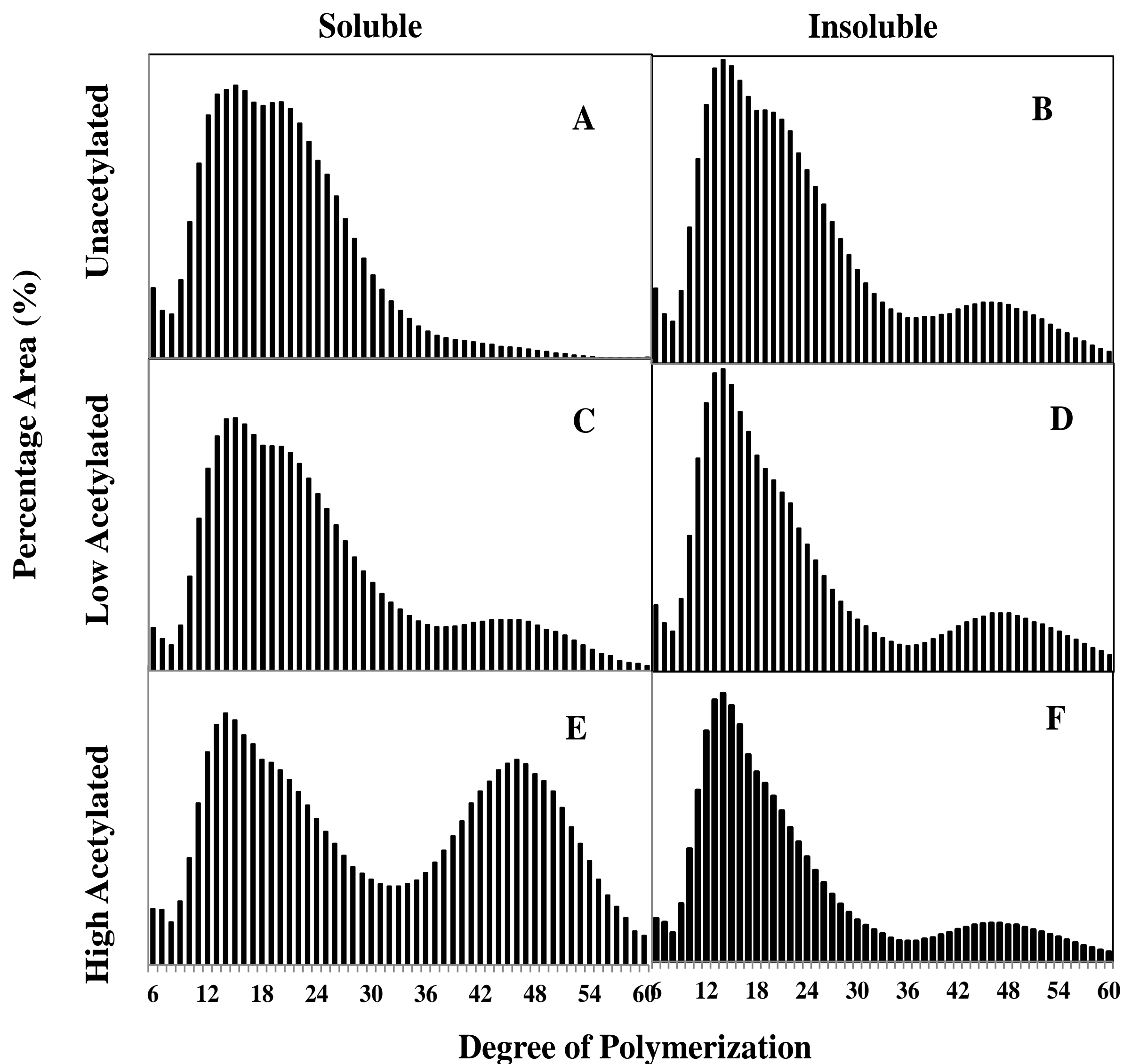

Figure 3. Normalized chain-length distributions of recovered starch chains from unacetylated and acetylated potato starches without β-amylase treatment after complexation.

The insoluble fractions of all starches with or without β-amylase treatment displayed a bimodal distribution with peak DP 13-14 and 43-45. These results indicate that upon complexation, different fractions of the starch chains form different types of complexes, that is, soluble or insoluble complex.

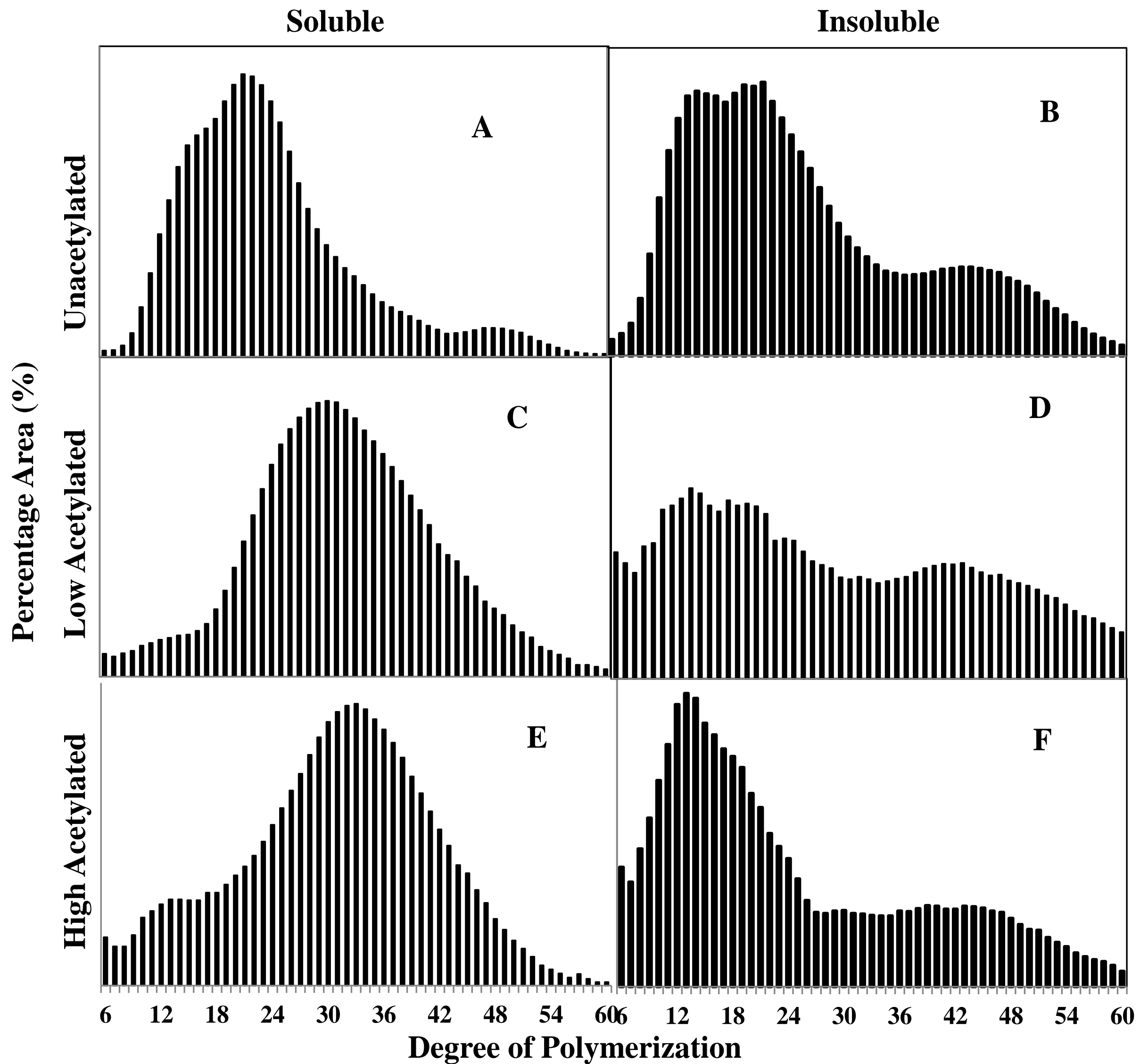

Figure 4. Normalized chain-length distributions of recovered starch chains from unacetylated and acetylated potato starches with β-amylase treatment after complexation.

When the amounts of complexed stearic acid (Table 2) were compared with the chain-length distributions (Figures 3 and 4), it was found that the DP range 20-43 from amylopectin chains might be more favorable for soluble complex formation, and the proportion of this DP range was increased after the β-amylase treatment. The first fraction of chains consisting of ~DP 6-17 present in all unacetylated and acetylated starches was decreased after β-amylase treatment. These results are in agreement with the molecular size distribution results in Figure 2 and indicate that DP 6-17 represent chains that are too short to participate in stearic acid complexation. The present results also agree with Eliasson,[30] who found that amylopectin chains could participate in complex formation because the favorable DP for complex formation was observed in all fractions.

Characterization of Starch-Stearic Acid Complexes

Melting Properties by Differential Scanning Calorimetry. The melting thermogram of starch-stearic acid complex was observed in all soluble and insoluble fractions except for the soluble fractions from the low and high acetylated starch without the β-amylase treatment (Table 3). This is in contrast to the stearic acid recovery results, which showed that stearic acid was recovered from all soluble and insoluble starch fractions, which will be discussed later in this section. All unacetylated starch-stearic acid complexes displayed peak melting temperatures (T_p) of 92.4-116.1 °C similar to those reported in previous studies,[25,31] which indicate the presence of both type I and II starch-stearic acid complex. The type I complex has a lower T_p of around 90 °C and is formed at lower temperatures of about 60 °C; thus, it is less ordered and exhibits low heat stability.[10,32] The type II complex has a T_p of around 110 °C[31] and is formed with continuous heat treatment and rearrangement of the type I complex at higher temperatures of about 90 °C .[15]

Table 3. Melting Temperatures and Enthalpies of Soluble and Insoluble Unacetylated and Acetylated Potato Starch-Stearic Acid Complexes[a].

starch	type of complex	β-amylase treatment	first peak				second Peak			
			T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)
Unacetylated	Soluble	No	86.5c (0.2)	92.4f (0.2)	97.1c (0.0)	0.67e (0.01)	99.8 (0.0)	116.1 (1.4)	127.1 (0.3)	3.53 (0.12)
	Insoluble	No	101.0a (1.9)	114.4a (1.2)	123.7a (0.0)	6.11c (0.62)				
	Soluble	Yes	92.2b (0.1)	110.2b (0.9)	124.7a (0.1)	3.61d (0.09)				
	Insoluble	Yes	94.1b (0.8)	107.9b (1.1)	122.0a (0.3)	9.68b (0.15)				
Low Acetylated	Soluble	No	ND[b]	ND	ND	ND§				
	Insoluble	No	79.2d (0.0)	101.1c, d (0.5)	114.6b (0.8)	13.92a (0.68)				
	Soluble	Yes	68.5e (0.1)	86.5g (0.6)	95.5c (2.0)	7.26c (0.23)	98.3 (3.5)	104.9 (3.4)	112.0 (5.6)	1.19 (0.45)
	Insoluble	Yes	77.7d (0.9)	102.2c (1.2)	113.9b (0.4)	15.29a (1.62)				

[a] Mean (standard deviation) of at least two measurements. Means in a column not sharing the same superscript letter are significantly different based on Tukey's honestly significant difference test ($p < 0.05$)

[b] ND- Not detected

Table 3. Melting Temperatures and Enthalpies of Soluble and Insoluble Unacetylated and Acetylated Potato Starch-Stearic Acid Complexes.[a] (Cont.)

starch	type of complex	β-amylase treatment	first peak				second Peak			
			T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)
High Acetylated	Soluble	No	ND	ND	ND	ND				
	Insoluble	No	77.5d	95.9e,f	110.5b	10.03b				
			(0.0)	(0.6)	(2.2)	(0.17)				
	Soluble	Yes	68.0e	82.8g	98.0c	6.54c				
			(0.9)	(0.8)	(0.9)	(0.13)				
	Insoluble	Yes	79.3d	97.6d.e	111.5b	13.30a				
			(0.9)	(1.8)	(0.3)	(0.08)				

[a] Mean (standard deviation) of at least two measurements. Means in a column not sharing the same superscript letter are significantly different based on Tukey's honestly significant difference test ($p < 0.05$)

[b] ND- Not detected

Although both type I and II starch-stearic acid complexes were present in the unacetylated starch complexes, the type II complex was more dominant as shown by its higher enthalpy value.

The low acetylated starch complexes exhibited a lower T_p than the unacetylated starch complexes for the same type of complex, and the T_p was further decreased with a higher DS of acetylation. This implies that for unacetylated starch, the nucleation rate for complexation was low, leading to sufficient propagation[10] and rearrangement of the complexes, and hence more ordered, heat-resistant complexes were formed. In contrast, the presence of acetyl groups may hinder the rearrangement of included helices and prevented the formation of the type II complex. The T_p values of all insoluble complexes were higher than their soluble counterparts for all acetylated starches; the high acetylated starch fractions did not always exhibit lower melting temperatures than their low-acetylated counterparts. Previous studies[13,28] have proposed that T_p of amylose-fatty acid complexes increased with an increase in amylose size. Therefore, the present results suggest that longer starch chains are responsible for the formation of insoluble complexes and shorter starch chains for the soluble complexes, supporting the previous HPSEC results (Figure 2).

More energy was required to disrupt the insoluble complex because it is composed of more highly organized or longer helices compared with the soluble complexes. Whittam et al.[33] reported that although the same energy was required to disrupt the helical complex structures of both crystalline and amorphous complexes of amylose and alcohols (chain length of four to eight carbon atoms), the crystalline complexes required an additional energy to break the crystal lattice that is absent in amorphous complexes. Hence this might explain why higher enthalpy values were observed for the insoluble complexes. Eliasson et al.[34] found that the enthalpy of an acetylated high-amylose maize starch-cetyltrimethylammonium bromide (CTAB) complex was

lower than that of the native high-amylose maize starch-CTAB complex. They concluded that acetylation of starch decreased its tendency to crystallize or form the starch-CTAB complex. However, in the present study, the enthalpy was higher in acetylated starches than the unacetylated starches for the same treatment. Later, Karkalas et al.[15] concluded that enthalpy values of amylose-lipid complexes should not be regarded as good indicators for measuring complex formation because precise results could not always be obtained because some samples could have polymorphs in the intermediate polymorphic state, or contain free amylose that could give false low-enthalpy readings[12].

According to Lui et al.,[27] a different type of supramolecular structure of complexes could exist in acetylated starch complexes due to the presence of acetyl groups. The presence of acetyl groups may promote the formation of a complex that is not crystalline in nature, which was supported by the absence of the endotherm for the soluble complexes of acetylated starches without the β-amylase treatment. However, when acetylation was combined with the β-amylase treatment, the endotherm of the soluble complex appeared, indicating that the presence of the nonacetylated portion of the amylose and amylopectin chains may interfere with the arrangement of the included stearic acid when the other portion was more acetylated as evidenced by the increased DS after β-amylase treatment (Table 1). Therefore, when the less acetylated portion was hydrolyzed by β-amylase, stearic acid was capable of better arranging itself within the starch helical structure, thus resulting in increased crystalline structure.

X-ray Diffraction Pattern. Except the unacetylated and without β-amylase treatment (Figure 5A) all insoluble starch complexes showed the V-type diffraction patterns with characteristic peaks at diffraction angle $2\theta = 7.4°$, 12.9º, and 20º (Figure 5). Debranched unacetylated starch complexes (Figure 5A and B) had additional peaks at $2\theta = 17.0°$, 22º, and 24º for soluble

complexes and at 2θ = 17.0º and 24º for insoluble complexes, which indicate the presence of retrogradation. These peaks were absent in all acetylated starch complexes because the acetyl groups hindered starch retrogradation except for the soluble low acetylated starch (Figure 5C). This may be due to the reassociation of longer chains with low DS of acetylation and not involved in stearic-acid complexation. For the unacetylated insoluble starches complexes, the additional β-amylase treatment increased the intensity of the V-type pattern and the peak at 2θ = 20.0º increased noticeably. These results agree with previous DSC enthalpy data (Table 3) that for insoluble complexes, the additional β-amylase treatment resulted in starch with chain lengths that favored interaction with stearic acid instead of reassociation. The characteristic peak for the starch-stearic acid complex 2θ = 20.0º was also observed in the soluble fractions of unacetylated starch complexes, indicating that native starches also formed soluble complexes, although only in small quantities. The present results again agree with previous results on the amount of complexed stearic acid (Table 2).

When low acetylation only was applied to starch, the X-ray diffraction peaks of the starch-stearic acid complex became predominant in the insoluble fraction, but the intensity of the peaks decreased at the high acetylation (Figure 5C,E). At high acetylation, the acetyl groups may interfere with proper arrangement of stearic acid in the complexes, thus resulting in complexes of low crystallites. These results support previous DSC results that for insoluble fractions, the low acetylated starch complex had higher enthalpy than the high acetylated one (Table 3). These results agree with Lui et al.,[27] who suggested that acetyl groups caused a reduction in the crystallinity of complexes and that acetylation resulted in complexes that existed mainly in the amorphous state rather than in the crystalline form.

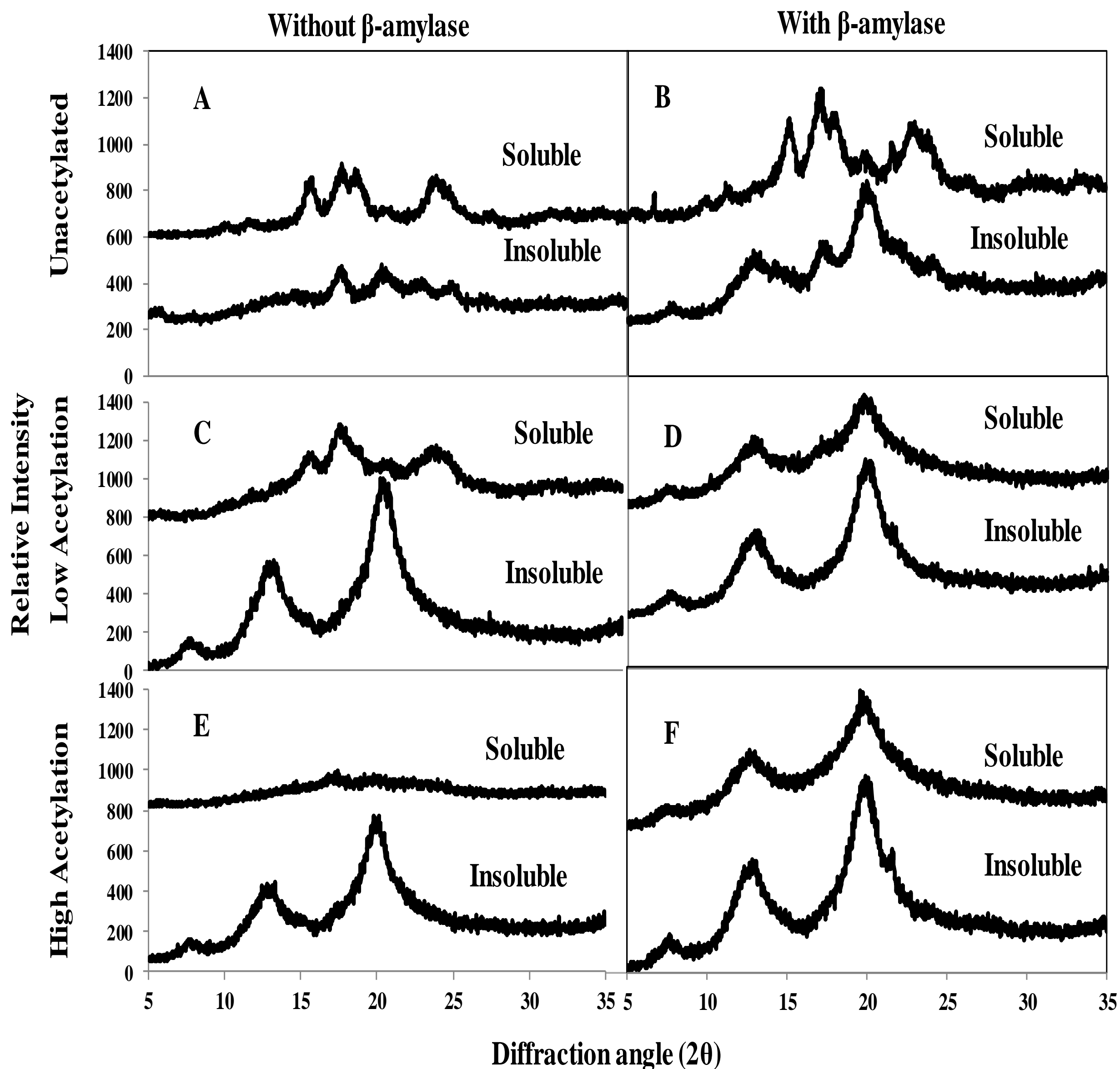

Figure 5. X-ray diffraction patterns of the soluble and insoluble fractions of debranched unacetylated and acetylated potato starches and their stearic acid complexes; (A) potato starch; (B) β-amylase-treated potato starch; (C) low acetylated potato starch; (D) low acetylated β-amylase treated potato starch; (E) high acetylated potato starch; (F) high acetylated β-amylase-treated potato starch.

The same trend was also noted for corresponding soluble complex fractions, in which peak intensity decreased for the high acetylated-only starch complex. The characteristic peaks of starch-stearic acid complex were barely noticeable in both acetylated-only soluble starch complexes, which support the DSC results (Table 3) and indicates that no crystalline structure was present in these soluble complexes. Nevertheless, the presence of an amorphous structure does not suggest the absence of a starch-stearic acid complex because it may still consist of unpacked helical amylose chains[28] as evidenced by the presence of complexed stearic acid (Table 2).

The V-type X-ray pattern was not evident for the soluble fractions until the β-amylase treatment was combined with acetylation (Figure 5D,F). This again supports previous DSC and DS findings that an optimum chain length of starch chains and high acetyl content encouraged the formation of crystalline soluble complex. In addition, when a combination of acetylation and β-amylase treatment was employed, the peak intensities increased with increasing acetylation in both the soluble and insoluble complexes (Figure 5D,F). However, in the debranched-only acetylated potato starch complexes (Figure 5C,D), the reverse was noted as the peak intensities of complexes decreased with increasing acetylation, which further emphasized the importance of starch chain length in complex formation.

Some insoluble complexes showed a small peak at $2\theta = 21.5^{\circ}$ (Figure 5D,F), which has been ascribed to pure stearic acid that is only physically trapped between starch helices[35] but not included in the starch helix cavity. Overall, the highest crystallinity for insoluble fractions was observed for low acetylated, debranched-only starch (Figure 5C), and for the soluble fractions, the highest crystallinity was observed for high acetylated, debranched, and β-amylase-treated starch (Figure 5F), which agree with the amount of complexed stearic acid quantified (Table 2).

CONCLUSIONS

In conclusion, the present results show that low acetylation encouraged the formation of complexed stearic acid with debranched potato starch for insoluble complexes, whereas high acetylation increased the amount of complexed stearic acid for soluble complexes. The acetylation reduced retrogradation, increased the hydrophobicity of the cavity, and ultimately encouraged more starch-stearic acid interaction. A combination of acetylation, debranching and β-amylase treatment was required to produce soluble, crystalline starch-stearic acid complex; soluble, amorphous starch-stearic acid complexes were formed in acetylated and debranched-only starches. Acetylation reduced melting temperature and increased the enthalpy values of all starch-stearic acid complexes compared with unacetylated ones. There was an optimum range of chain length to encourage the formation of starch-stearic acid complexes. Information on the generation and properties of soluble starch-stearic acid complex from this study may provide insight into the creation of soluble complexes between starch and other insoluble bioactive compounds and may pave the way for applications whereby soluble complexes are desired to improve bioavailability.

ABBREVIATIONS USED

DS, degree of substitution; DP, degree of polymerization; DSC, differential scanning calorimetry; HPSEC, high-performance size exclusion chromatography; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed-amperometric detection; GC, gas chromatography

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APPENDIX

Authorship Statement for Chapter 3

I verify that Emily Arijaje is the first author and completed over 51% of the research work published in the following article that is included in her dissertation: Effects of Chemical and Enzymatic Modifications on Starch-Stearic Acid Complex Formation

Dr. Ya-Jane Wang

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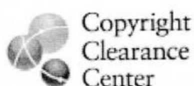

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VI. Chapter 4 : Effects of Chemical and Enzymatic Modifications on Starch-Oleic Acid Complex Formation

ABSTRACT

The solubility of starch-inclusion complexes affects the digestibility and bioavailability of the included molecules. Acetylation with two degrees of substitution 0.041 (low) and 0.091 (high) combined without or with a β-amylase treatment were employed to improve the yield and solubility of inclusion complex between debranched potato starch and oleic acid. Both soluble and insoluble complexes were recovered and analyzed for their degree of acetylation, complexation yields, molecular size distributions, X-ray diffraction patterns and thermal properties. Acetylation significantly increased the amount of recovered soluble complexes as well as the complexed oleic acid in both soluble and insoluble complexes. High acetylated-only starch complexed the highest amount of oleic acid (38.0 mg/g) in the soluble complexes; low acetylated starch with or without the β-amylase treatment resulted in the highest complexed oleic acid in the insoluble complexes (37.6 - 42.9 mg/g). All acetylated starches displayed the V-type X-ray pattern, and the melting temperature generally decreased with acetylation. The results indicate that starch acetylation with or without the β-amylase treatment can improve the formation and solubility of starch-oleic acid complex.

KEYWORDS: acetylation, modified starch, starch-oleic acid complex, β-amylase, soluble complex, insoluble complex,

INTRODUCTION

Amylose forms a helical structure with a hydrophobic cavity that can react with hydrophobic molecules such as iodine,[1] alcohols,[2] ibuprofen,[3] salicylic acid,[4] genistein,[5] flavors,[6] and fatty acids[7-15] to form inclusion complexes. Fatty acids are commonly employed as model molecules to elucidate the optimum conditions required for complex formation because of their varying chain lengths and structures. Upon forming inclusion complex, the included molecule is stabilized and protected from degradation,[6,16] oxidation,[15,17-18] light, and high temperature.[5] Inclusion complex can also serve as a vehicle for controlled release of the included molecule.[15,17-19] Nevertheless, when inclusion complexation occurs, there is an increase in the crystallinity and subsequent precipitation of the complex. The precipitation reduces solubility and hydrolysis of the complex, and can eventually limit bioavailability of the included molecule.

Most studies on starch-inclusion complexes focus on insoluble complexes, and limited information is available on the formation of soluble complexes by using modified starch. Wulff and Kubik[20] found that hydroxypropylation of amylose with a degree of substitution (DS) 0.075 was sufficient to produce soluble complexes with sodium dodecyl sulfate (SDS). Acetylation of pea starch yielded amorphous insoluble complexes with lauric acid and reduced the amount of insoluble complexes recovered.[21] Acetylation of high amylose maize starch decreased its complexing ability with cetyltrimethylammonim (CTAB) when studied by differential scanning calorimetry.[22] Our previous work[23] demonstrated that acetylation reduced the formation of insoluble complexes between debranched potato starch and stearic acid by 35-52% depending on the degree of acetylation, but increased the yield of soluble complexes by 8.3% for both low and high acetylated starches. When the acetylated debranched starches were reduced in molecular size by β-amylase, the formation of soluble complexes with stearic acid was improved by 154-

245% when compared with its unacetylated counterparts. The highest amount of complexed stearic acid (123.1 mg/g) was recovered from the low-acetylated β-amylase-treated insoluble complex; the highest amount of stearic acid in the soluble complexes (61.2 mg/g) was obtained from the high-acetylated β-amylase-treated starch. This study used the same chemical (acetylation) and enzymatic (isoamylase and β-amylase) modifications to investigate the formation of soluble and insoluble complexes between starch and oleic acid (C18:1). The impacts of the presence of one double bond in oleic acid on the yield and properties of soluble and insoluble complexes with modified starch were investigated.

MATERIALS AND METHODS

Materials. Potato starch was obtained from Penford Food Ingredients (Centennial, CO, USA) and used without further treatment. Isoamylase from *Pseudomonas sp* (specific activity 280 units/mg protein), Pullulanase from *Klebsiella planticola* (specific activity 34 units/mg protein) and β-amylase from *Bacillus cereus* (specific activity 2660 units/mg protein) were purchased from Megazyme International Ireland Ltd. (Wicklow, Ireland). Oleic (*cis*-9-Octadecenoic) acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of ACS grade.

Acetylation of Starch. Two levels of acetylation (low and high) of starch was carried out according to the method by Wang and Wang.[24] The acetyl content was determined according to the method of McComb and McCready[25] and expressed as degree of substitution (DS).[26]

Enzymatic Modification of Starch. ***Debranching.*** Starch (15 g wet basis) was added to 400 mL water (3.75% w/v) and gelatinized in a boiling water bath for 1 h with constant stirring. The temperature of the solution was equilibrated to 45 °C and the pH adjusted to 5.0 with 0.5 M HCl.

To the starch solution, isoamylase and pullulanase each at 1.33% (v/w, starch db) was added, and then incubated at 50 °C with constant stirring for 48 h. The starch was recovered with 4-fold volume of pure ethanol, centrifuged at 7000*g* for 10 min, dried at 40 °C for 48 h and milled using a UDY cyclone mill (UDY Corp., Ft. Collins, CO, USA) fitted with a 0.5-mm screen.

β-Amylase Treatment. A portion of the debranched starch was further subjected to β-amylase hydrolysis to reduce the degree of polymerization (DP). After debranching for 48 h, the starch slurry pH was adjusted to 6.5 with 0.5 M NaOH, and incubated with 0.5% (v/w, starch db) β-amylase at 40 °C for 4 h. The enzyme reaction was terminated by boiling for 15 min. The β-amylase-treated starch was recovered as previously described.

Characterization of Starch Structure. The molecular size distributions of debranched unacetylated and acetylated starch without or with the β-amylase treatment were recovered after complexation with oleic acid and characterized using a high-performance size exclusion chromatography (HPSEC) system (Waters Corp., Milford, MA, USA). Starch (10 mg) was dissolved in 5 mL of 90% DMSO, boiled for 1 h, and filtered through a 5.0-μm filter prior to injection into the HPSEC system. The HPSEC system consisted of a guard column (OHpak SB-G, 6.0 × 500 (mm) i.d. × length), two Shodex columns (OHpak KB-804 and KB-802, both 8.0 × 300 (mm) i.d. × length), a 200 μL injector valve (model 7725i, Rheodyne, Cotati, CA, USA), an inline degasser, a model 515 HPLC pump, and a model 2414 refractive index detector. The mobile phase of 0.1 M sodium nitrate with 0.02% sodium azide was eluted at a flow rate of 0.6 mL/min. The temperature of column was maintained at 60ºC and the detector at 40ºC. Dextran standards of molecular weight of 5,200, 11,600, 23,800, 48,600, 273,000 and 410,000 g/mole from Waters Corp. (Milford, MA, USA) and 1,050,000 g/mole from Sigma Aldrich (St. Louis, MO, USA) were used to establish the calibration curve.

The amylopectin chain length distribution was characterized by high-performance anion-exchange chromatography equipped with pulsed-amperometric detection (HPAEC-PAD) according to the method of Wong and Jane.[27] The chains were divided into DP ranges and classified as follows: A chains (DP 6-12), B1 chains (DP 13-24), B2 chains (DP 25-36), and B3+ chains (DP 37+).[28] The average chain length was calculated as the cumulative sum of the product of DP and percentage relative areas for all the identified peaks.

Complexation of Starch and Oleic Acid. All samples were prepared in replicates. The starch solution (3.75% w/v), debranched or debranched and β-amylase treated, was adjusted to pH 7.0, equilibrated to 80 °C, and mixed with 1 g of oleic acid dissolved in warm 95% ethanol. The mixture was maintained at 80 °C for 30 min and stirred continuously to ensure that there was sufficient interaction between starch and oleic acid, and then the temperature was maintained at 45 °C overnight with continuous stirring. The resulting mixture was centrifuged at 7000*g* for 10 min, from which the precipitate, that is, "*insoluble complex*", was obtained, whereas, the "*soluble complex"* was recovered by precipitating the supernatant with 4-fold volume of pure ethanol. Any uncomplexed oleic acid was removed from both the *insoluble* and *soluble* complexes by rinsing excess 95% ethanol. Complexes were rotated in excess 95% ethanol using a labquake shaker rotisserie (Barnstead/Thermolyne, Dubuque, IA, USA) at room temperature for 2 h, centrifuged at 7000*g* for 10 min, dried at 40 °C for 48 h, milled using a mortar and pestle, sieved through a 250-µm sieve, and stored for further analysis.

Hydrolysis of Complexes and Oleic Acid Analysis. Soluble or insoluble complex (100 mg) was added with 10 mL of 1 M HCl and heated with continuous stirring in a boiling water bath for 1 h. After the complex mixture was cooled, 5 mL hexane was added, and the solution was rotated on the rotary shaker for 1 h. The hexane layer with the extracted oleic acid was

recovered, and the extraction was repeated with another 5 mL hexane for 1 h. To the recovered hexane phase, boron trifluoride methanol was added to convert oleic acid to oleic acid methyl esters. An internal standard of methyl heptadecanoate (~1 mg) was subsequently added to all samples. The oleic acid methyl ester was injected into a gas chromatographer (GC) (GC-2010, Shimadzu, Kyoto, Japan) equipped with a BP 21 capillary column (30 m × 0.25 mm i.d.; SGE Inc., Austin, TX) with a flame ionization detector (FID), and responses were collected by Shimadzu GCsolution Workstation 2.3 (Kyoto, Japan). The injection port and detector temperatures were set at 220 °C and 230 °C, respectively. The column oven temperature was equilibrated at 100 °C for 1 min, ramped up at 15 °C /min to 160 °C, again ramped up at 5 °C /min to 200 °C and maintained at 200 °C for 10 min. The flow rate of the carrier gas (helium) was 30 mL/min. The concentration of oleic acid was determined from a standard curve prepared by using oleic acid methyl ester solution (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL) containing the internal standard of methyl heptadecanoate (0.5 mg/mL).

Physicochemical Properties. A diffractometer (PW1830 MPD, Philips, Almelo, The Netherlands) was used to determine the powder X-ray diffraction (XRD) pattern of complexes. The generator voltage was set at 45 kV and the current at 40 mA. The sample was scanned over the 2θ angular range from 5° to 35° with a step size of 0.02° and time of 1 s per step.

The thermal stability of all complexes was measured using a differential scanning calorimeter (DSC, Pyris-Diamond, PerkinElmer, Shelton, CT, USA). Approximately 8 mg of complex was weighed into a stainless steel pan, 16 µL of distilled water was added with the aid of a microsyringe, and the pan was hermetically sealed. The sample was equilibrated for 24h at room temperature before scanning and were scanned from 25 to 180 °C at 10 °C/min, immediately cooled from 180 to 25 °C at 40 °C/min and rescanned from 25 to 180 °C at 10

°C/min to confirm the formation of the starch-fatty acid complex. The onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c) and enthalpy (ΔH) of the endotherms were calculated using the Pyris data analysis software.

Statistical Analysis. All experiments were conducted in replication, and data were analyzed using JMP software (SAS Institute Inc., Cary, NC, USA). The means were compared using Tukey's honestly significant differences (HSD) test.

RESULTS AND DISCUSSION

Degree of Substitution. The low and high acetylated starches used for complex formation with oleic acid had a DS of 0.041 and 0.091, respectively (Table 1). Acetylated starches were then debranched with isoamylase and treated with or without β-amylase prior to complexation. After complexation the soluble and insoluble complexes were recovered and determined for their DS of acetylation. Similar to the previous study,[23] the soluble complex fractions had a significantly higher DS than the insoluble ones for the same treatments. When β-amylase was combined with acetylation, an increase in DS was observed for all soluble and insoluble complexes. The lowest DS of acetylated starch to form soluble complex with stearic acid was 0.063 in our previous study,[23] however, oleic acid formed soluble complexes at a lower DS of 0.045 in the present study. In addition, the difference in DS of acetylation between the soluble and insoluble complexes of oleic acid was significantly smaller than that of stearic acid. These differences in DS suggest that a lower DS of acetylation on starch was sufficient to encourage soluble complex formation with more polar molecules, such as oleic acid, compared with less polar molecules, such as stearic acid.

Table 1. Degree of Substitution (DS) of Acetylated Starches and Oleic Acid Complexes[a].

starch	**β-amylase treatment**	**type of complex**	**DS**
low acetylated starch	N/A	N/A[b]	0.041±0.001d
low acetylated starch complex	no	soluble	0.045±0.005d
		insoluble	0.036±0.005d
	yes	soluble	0.071±0.000c
		insoluble	0.045±0.001d
high acetylated starch	N/A	N/A	0.091±0.001b
high acetylated starch complex	no	soluble	0.077±0.002b,c
		insoluble	0.062±0.004c
	yes	soluble	0.119±0.004a
		insoluble	0.072±0.009c

[a]At least replicate samples were prepared for each complex. Data of two measurements with standard deviation. Means in a column not sharing the same letter are significantly different based on Tukey's honestly significant difference test ($p < 0.05$).
[b]N/A, not applicable.

Complex Recovery and Complexation Yield. Complex recovery was determined by comparing the individual recovered complex weight against the initial material weight. The highest recovery for the insoluble complexes was 0.73 g/g from the unacetylated β-amylase treated starches (Table 2), which is similar to results from previous studies.[6,15,23] The total recovery when both soluble and insoluble complexes were included was 0.92-0.96 g/g, except for the unacetylated β-amylase treated starch of a total recovery of 0.85 g/g. These results indicate that modification of starch prior to complex formation did not change the total recovery but changed the proportions

Table 2. Complex Recovery and Percentage Oleic Acid Content Recovered from All Complexes[a].

starch	type of complex	β-amylase treatment	recovery (g/g)	oleic acid in individual complex (mg/g)
unacetylated	soluble	no	0.34±0.04c,d	3.0±0.3g
	insoluble	no	0.61±0.02a,b	3.3±0.1g
	soluble	yes	0.12±0.01e	10.9±1.0e
	insoluble	yes	0.73±0.01a	4.8±0.0f
low acetylated	soluble	no	0.56±0.03b	16.3±0.1c,d
	insoluble	no	0.39±0.03c,d	37.6±3.6a
	soluble	yes	0.40±0.04c	26.3±0.3b
	insoluble	yes	0.56±0.01b	42.9±6.0a
high acetylated	soluble	no	0.66±0.01a,b	38.0±2.2a
	insoluble	no	0.27±0.01d	20.1±0.5b,c
	soluble	yes	0.57±0.08b	12.5±1.1d,e
	insoluble	yes	0.35±0.01c,d	20.9±1.0b,c

[a]Data of at least two measurements with standard deviation. Means in a column not sharing the same letter are significantly different based on Tukey's honestly significant difference test ($p < 0.05$).

of the soluble and insoluble complexes for most complexes. The recovery of the insoluble complexes decreased with increasing DS of acetylation, whereas the opposite trend was observed for the soluble complexes. Acetylation increased the recovered soluble complexes by 65 and 94% for low and high acetylated starches, respectively, compared with the unacetylated one.

When an additional β-amylase treatment was incorporated, the recovery of the soluble complexes was decreased or unchanged, whereas that of the insoluble complexes was increased or unchanged. These results imply that the length of starch chains present in the debranched-only starches were more favored for the formation of soluble complexes. The β-amylase treatment

hydrolyzed some starch chains to a length that was too short to form complex, thus resulting in a decrease in the proportion of recovered soluble complexes. Within the β-amylase-treated starches, the recovery of the soluble complexes was increased by 233 to 375% for low and high acetylated starches, respectively, when compared with the unacetylated starch.

The oleic acid content in all starch complexes was determined by GC (Table 2). Acetylated starches resulted in significantly higher amounts of complexed oleic acid per gram of complex in the recovered complexes compared with unacetylated starches for most treatments, suggesting that acetylation encouraged the formation of both soluble and insoluble complexes between starch and oleic acid. However, the amounts of complexed oleic acid were significantly lower than those of complexed stearic acid reported in the previous work.[23] This is attributed to a better stability of the complexes formed between starch and stearic acid, a saturated fatty acid, than oleic acid of an unsaturated fatty acid.[8,10,29-30]

For the insoluble complexes, the highest amount of oleic acid per gram of complex was recovered from the low acetylated starch (37.6 - 42.9 mg), which also complexed the higher amounts of stearic acid (101.0 and 123.1 mg) in the previous study.[23] This indicates that the presence of a low level of acetylation (DS 0.036 - 0.045) hindered starch retrogradation and encouraged complexation with oleic acid. However, a high level of acetylation (DS 0.062 – 0.072) decreased not only the complex recovery but also the complexation yield with oleic acid. This indicates that the further increase in the number of acetyl groups on starch might destabilize the starch helices for complexation. In addition, when β-amylase treatment was combined with high acetylation of starch, the amount of complexed oleic acid remained unchanged, suggesting that with the β-amylase treatment of high acetylated starch, there were still starch chains with favorable lengths that could participate in complexation with oleic acid.

For the soluble complexes, the highest amount of oleic acid was recovered from high acetylated-only starch (38.0 mg), which was similar to the highest amount of complexed oleic acid in the insoluble complexes. In contrast, the highest amount of stearic acid was recovered from the high acetylated and β-amylase-treated starch (61.2 mg).[23] It is hypothesized that the cis double bond structure of oleic acid might require a slightly longer starch chains than stearic acid to form soluble complexes with high acetylated starch.

Molecular Size Distribution. The standard curve used to estimate the degree of polymerization of debranched starch complexes without or with the β-amylase treatment is displayed in Figure 1.

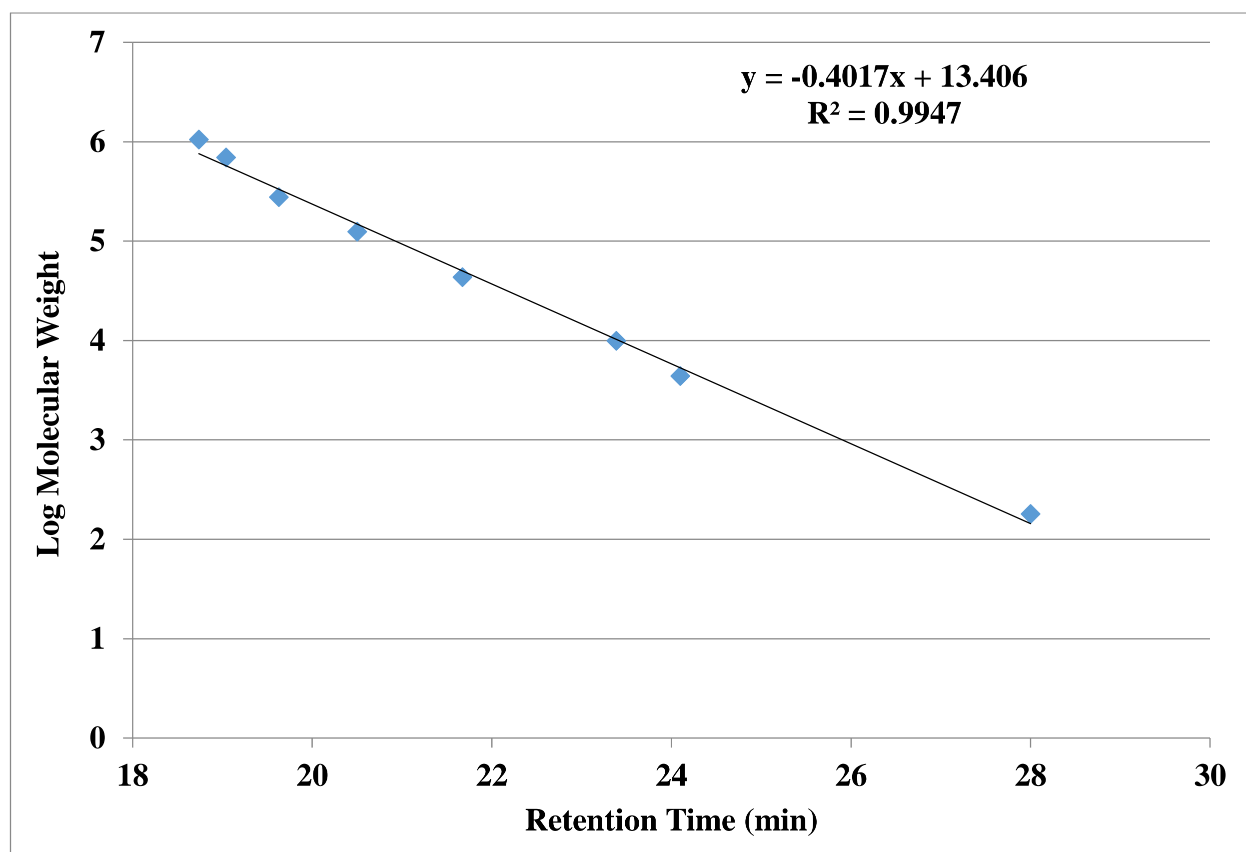

Figure 1. Standard curve used to estimate the degree of polymerization of debranched starch complexes without or with the β-amylase treatment.

Except for the high acetylated-only starch (Figure 2E), all recovered starch in the starch complexes displayed an amylose peak at a retention time of 18.2-18.5 min, which corresponded to DP ~7000 (Figure 2). The relative proportion of the amylose peak was greater in the insoluble complexes than in the soluble complexes and increased with increasing acetylation level. This increasing trend in the amylose peak was also noted in the previous study with stearic acid,[23] which was attributed to an increased hydrodynamic volume from higher acetyl contents.

The peak DP of soluble complexes shifted to a higher DP for unacetylated and acetylated starches after the β-amylase treatment, indicating that the β-amylolysis of starch hydrolyzed short chains and reduced the chain length of longer chains. For the insoluble complexes, the DP remained unchanged for unacetylated and low acetylated starch and slightly increased for the high acetylated starches after the β-amylase treatment.

All complexes displayed a main peak at a retention time between 23 and 24 min, which corresponded to DP 91 and 36, respectively, and were composed of mainly amylopectin long branched chains that were assumed to be involved in the complex formation with oleic acid. The retention time of this peak shifted to a shorter time with increasing acetylation, which was similar to the amylose peak. The relative proportion of this main peak was greater in the soluble complex than in the insoluble complex for the same treatment. These results are similar to those in the previous stearic acid study, although a lower amount of oleic acid was complexed in the present study.

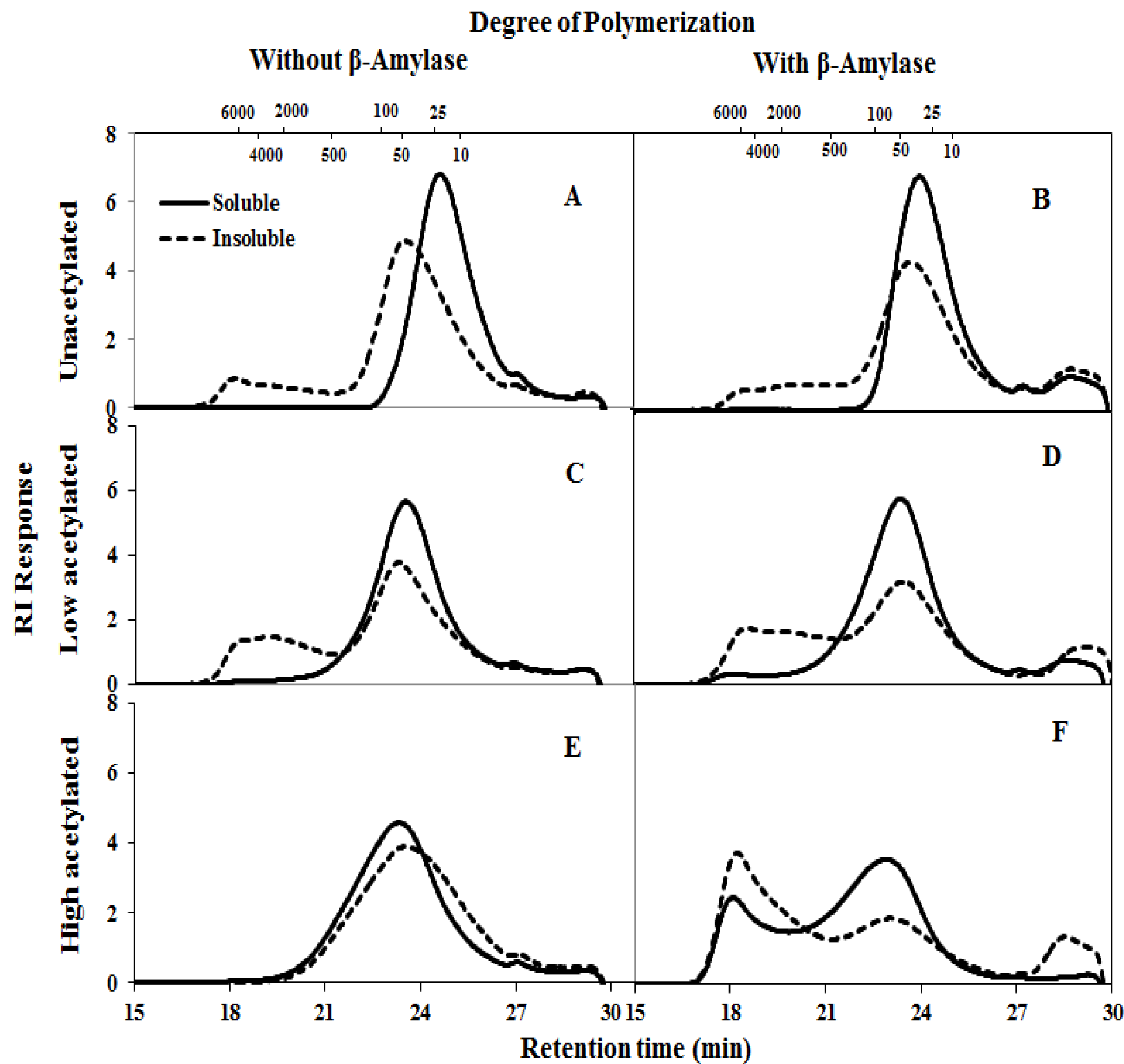

Figure 2. Normalized size-exclusion chromatograms of recovered soluble and insoluble starch complexes from unacetylated and acetylated potato starches after complexing with oleic acid: (A) potato starch; (B) β-amylase treated potato starch; (C) low acetylated potato starch; (D) low acetylated β-amylase treated potato starch; (E) high acetylated potato starch; (F) high acetylated β-amylase treated potato starch.

Although previous studies reported that the carbon atoms adjacent to the double bond in cis-unsaturated fatty acids may be capable of rotating freely to give rise to a relatively linear structure,[10,31] the complexation of starch with stearic acid was still more favored than with oleic acid. Overall, starch fractions with DP between 14 and 363, corresponding to retention time 25 and 21.5 min, respectively, were observed in all starch complexes, indicating that these starch chain lengths were primarily responsible for complex formation with oleic acid. This agrees with Gelders et al[32] that amylose chains with DP up to 400 were effective in forming complexes with glyceryl monostearate (GMS) and docosanoic acid (C22).

The chain-length distributions of starch chains up to DP 60 of all starch complexes without and with the β-amylase treatment are displayed in Figures 3 and 4, respectively. For the soluble complexes, the additional β-amylase treatment resulted in an increase in the peak DP and the proportion of the higher DP peak, but it did not change the profiles of the insoluble complexes as much. The peak DPs for the soluble complexes of acetylated starches in the present work were slightly higher but not significantly different from those reported in the starch-stearic acid study,[23] although a higher amount of stearic acid was complexed than oleic acid. Similar to the starch-stearic acid study,[23] the fraction of DP 6-17 was prominent in all starch complexes, but it represented the proportion of starch chains that were too short to form complexes.

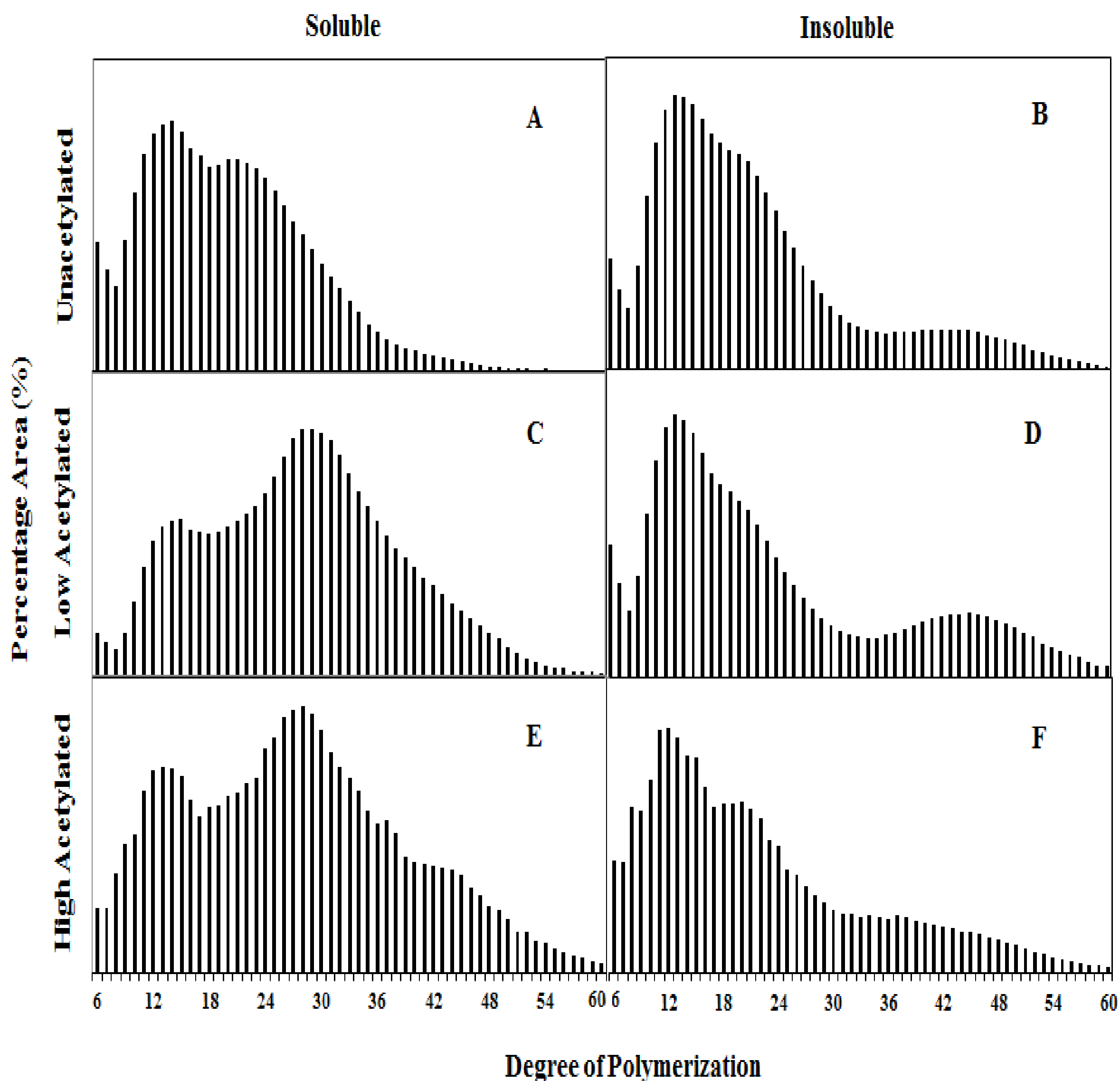

Figure 3. Normalized chain-length distributions of recovered soluble and insoluble starch complexes from unacetylated and acetylated potato starches without the β-amylase treatment after complexing with oleic acid using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD): (A) potato starch; (B) β-amylase treated potato starch; (C) low acetylated potato starch; (D) low acetylated β-amylase treated potato starch; (E) high acetylated potato starch; (F) high acetylated β-amylase treated potato starch.

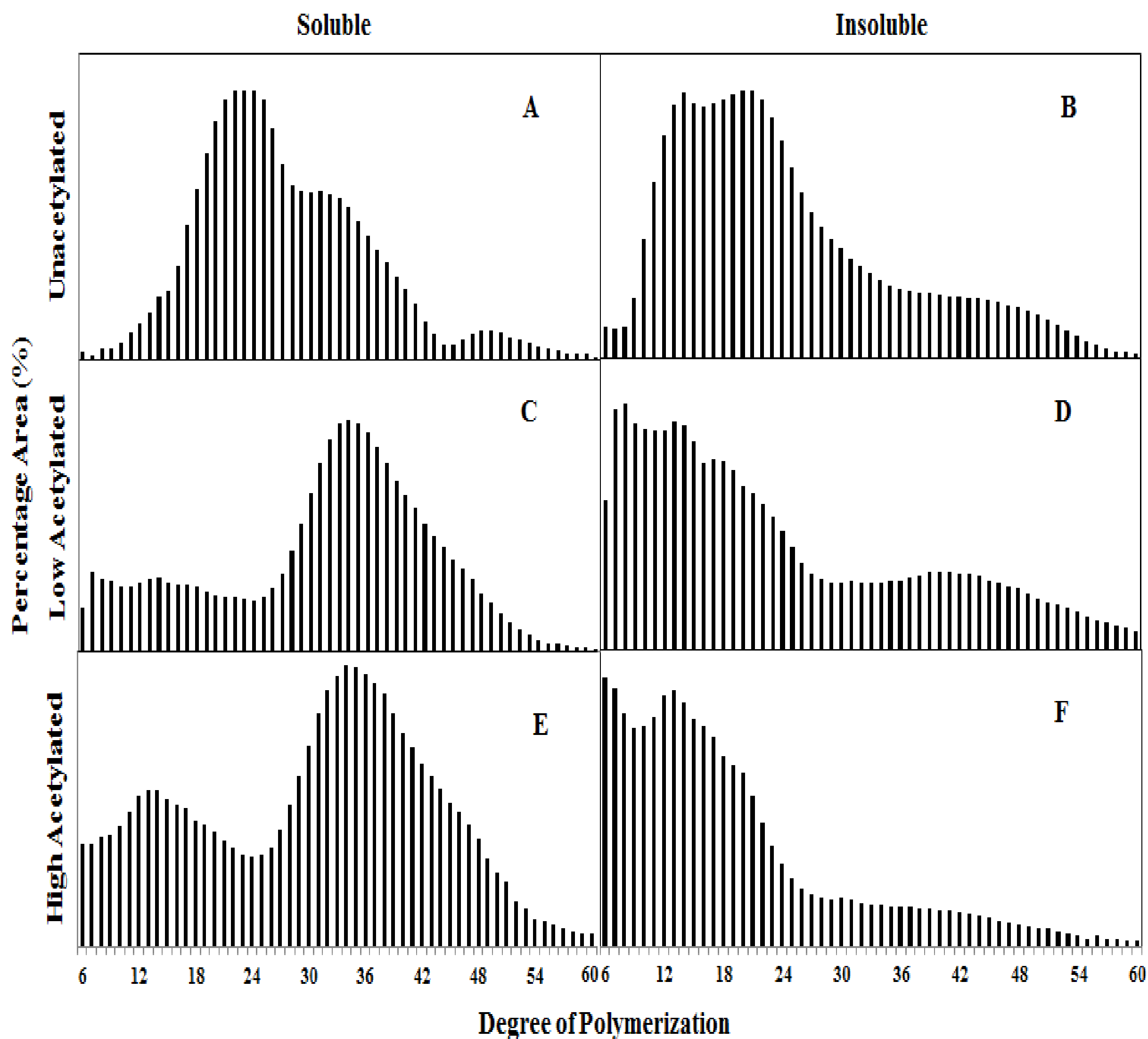

Figure 4. Normalized chain-length distributions of recovered soluble and insoluble starch complexes from unacetylated and acetylated potato starches with the β-amylase treatment after complexing with oleic acid using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD): (A) potato starch; (B) β-amylase treated potato starch; (C) low acetylated potato starch; (D) low acetylated β-amylase treated potato starch; (E) high acetylated potato starch; (F) high acetylated β-amylase treated potato starch.

The proportion of DP 6-17 decreased with the β-amylase treatment for the soluble complexes but increased slightly for the insoluble complexes. These results indicate that when the amylopectin chains were considered, the favorable chain length for complexing with fatty acid, which was reported[9] as DP >20, was longer for the soluble complexes than for the insoluble complexes.

Characterization of Starch-Oleic Acid Complexes

X-ray Diffraction Pattern. Most soluble and insoluble complexes of unacetylated starches displayed a mixture of the B-type and V-type X-ray diffraction patterns (Figure 5A,B) with peaks at 2θ = 17.0º, 22º and 24º and 2θ = 12.9º and 20º, respectively. The only exception was the soluble complex from the unacetylated-only starch (Figure 5A), which displayed predominantly the A-type pattern with peaks at 2θ = 15.3º, 17.2º, 18.2º and 23.1º besides minor peaks at 2θ = 10º, 11.5º, 20º and 26.5º from the V-type pattern. The presence of the A-type polymorph may be ascribed to the high proportion of amylopectin short chains (Figure 3A) that were too short to complex with oleic acid but prone to re-association to form very ordered structure[33]. The additional β-amylase treatment on unacetylated starch decreased the intensity of the V-type pattern for the insoluble complexes, implying that the formation of insoluble starch-oleic acid complexes may require longer starch chains than starch-stearic acid complexes.[23] The soluble complexes of the unacetylated starch changing from the A-type to B-type pattern after the β-amylase treatment supports the HPSEC results that shorter chains have been hydrolyzed to result in predominantly longer chains. This is in agreement with previous works in which starches with shorter average chain length exhibited the A-type pattern (DP <19.7) and starches with a longer average chain length (DP >21.6) exhibited the B-type pattern.[34] All soluble and insoluble

acetylated complexes displayed the V-type pattern with peaks at 2θ = 7.4°, 12.9° and 20° (Figure 5C-F).

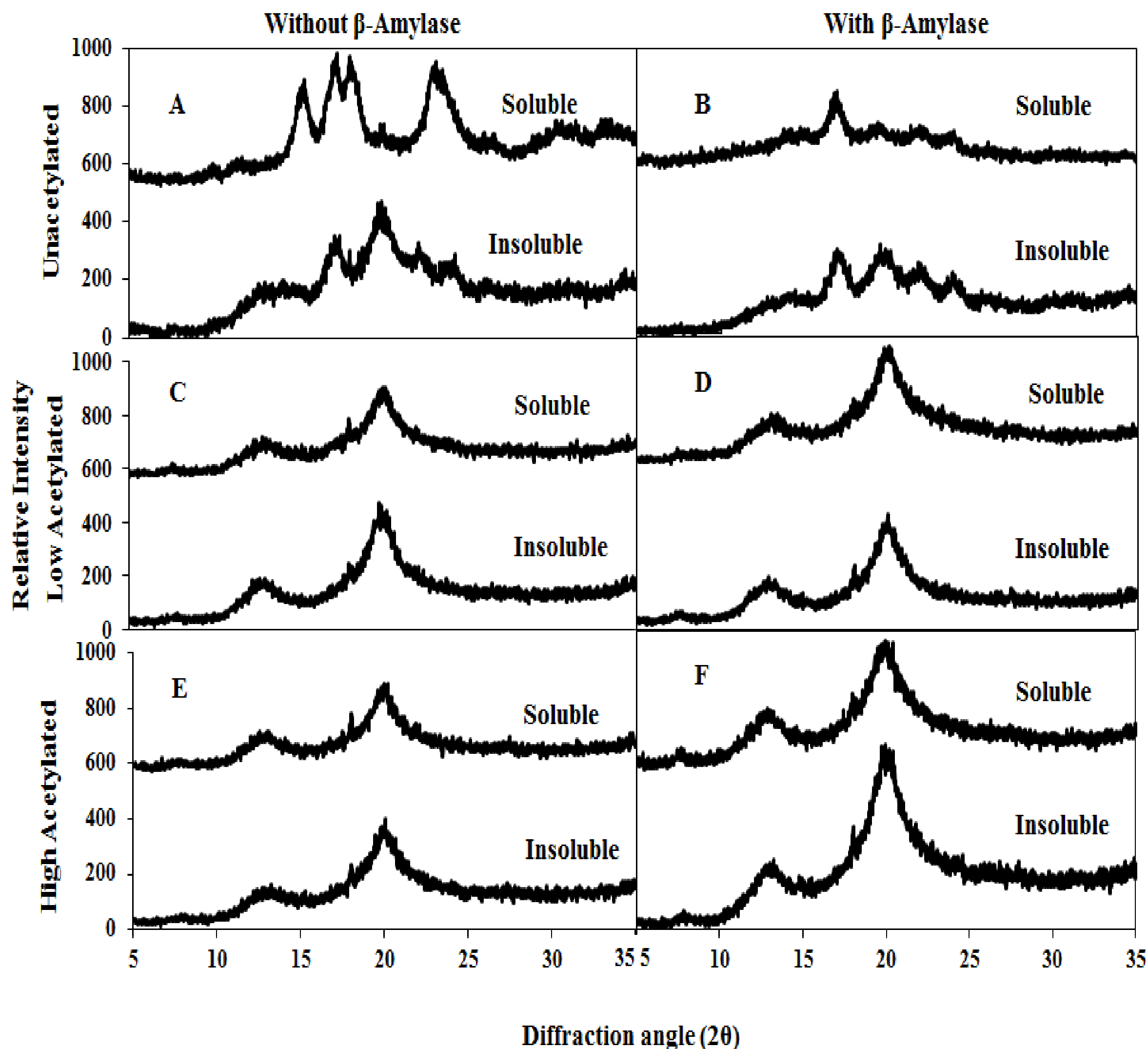

Figure 5. Normalized X-ray diffractograms of recovered soluble and insoluble starch complexes from unacetylated and acetylated potato starches after complexing with oleic acid: (A) potato starch; (B) β-amylase treated potato starch; (C) low acetylated potato starch; (D) low acetylated β-amylase treated potato starch; (E) high acetylated potato starch; (F) high acetylated β-amylase treated potato starch.

In the previous work of starch-stearic acid complexes[23], the V-type structure was not observed in the soluble complexes from acetylated-only starches, even though a significant quantity of stearic acid was detected by GC. The XRD results imply that soluble starch-oleic acid complexes were formed, but when the GC results (Table 2) were considered, these complexes did not include as much oleic acid as did the soluble complexes of stearic acid from the previous work. The reason for the difference in the XRD patterns observed in the two studies may be due to that stearic acid and oleic acid interacted differently with the acetylated starch helix because of their structural differences. However, limited information exists for inclusion complexes from chemically modified starch; therefore more research is needed to explain the differences between XRD patterns and complexed amount for acetylated starch complexes with stearic acid and oleic acid.

Acetylation of starch noticeably increased the intensity of the V-type pattern of starch-oleic acid complex and reduced starch retrogradation peak at $2\theta = 17^{\circ}$. For the insoluble complexes, high acetylated β-amylase treated starch displayed the highest peak intensity (Figure 5F), which, however, did not contain the highest amount of complexed oleic acid by GC. This suggests that the intensity of the V-type pattern might not necessarily correlate with the quantity of complexed fatty acids such as oleic acid in this study and stearic acid in the previous study, but only reflect the level of organization and arrangement of the type of complex formed. When low acetylated starches were treated with β-amylase, the peak intensity of the starch-oleic acid complex slightly increased for the soluble complexes but decreased for insoluble complexes (Figure 5C,D). Again, there was no correlation between X-ray peak intensity and the amount of complexed oleic acid.

Melting Properties by Differential Scanning Calorimetry. All insoluble complexes and some soluble complexes displayed melting endotherms, but only the insoluble complexes exhibited the type II complex peak (Table 3). Within the unacetylated starches, the insoluble complexes of the debranched-only starch displayed a single melting endotherm with T_p at 106.7 °C, signifying the presence of the type II starch-fatty acid complex, whereas, the soluble complex within the same treatment displayed only type I complex with T_p at 94.8 °C. The type I complex has been reported to form at a lower temperature of ~60 °C, leading to the production of randomly oriented helical segments,[35] while the type II complex is formed at temperatures around 90 °C and produces well defined crystallites.[36] Godet et al. [9,37] showed that longer starch chains can complex more lipids to produce crystals with higher melting temperatures.

When the unacetylated starch received the additional β-amylase treatment, two melting endotherms were observed for the insoluble complexes, including the type I and type II complex[38] with T_p at 94.9 and 102.9 °C, respectively. Lagendijk and Pennings[39] previously reported that amylose with DP 900 may contain between 10 and 12 helices, consisting of six glucose units per turn, and each helix can complex at least one glycerol monopalmitate. The bimodal distribution of the insoluble starch complexes consisting of both short and long starch chains (Figure 2B) may lead to production of the type I and type II complex, respectively, which is in agreement with previous works.[37,39] However, although the type II complexes were formed for the unacetylated starch complexes, the amount of complexed oleic acid were still low, presumably because of the increased reassociation of starch chains as evidenced in the XRD results (Figure 5B).

Table 3. Melting Temperatures and Enthalpies[a] of Recovered Soluble and Insoluble fractions of Unacetylated and Acetylated Potato Starch-Oleic Acid Complexes[b].

starch	type of complex	β-amylase treatment	type I complex peak				type II complex peak			
			T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)
unacetylated	soluble	no	88.7a,b (0.1)	94.8a,b (0.9)	101.2a (0.6)	1.59b,c (0.13)				
	insoluble	no	ND	ND	ND	ND	92.1 (0.5)	106.7 (0.0)	114.7 (0.7)	1.89 (0.03)
	soluble	yes	90.5a (0.6)	96.1a (0.7)	102.1a (0.5)	1.84b (0.10)				
	insoluble	yes	91.6a (0.8)	94.9a,b (0.8)	98.0a (1.7)	0.44d (0.10)	101.0 (0.9)	102.9 (1.1)	109.0 (0.5)	0.76 (0.06)
low acetylated	soluble	no	ND[c]	ND	ND	ND				
	insoluble	no	79.8c (0.7)	91.4b (0.4)	100.8a (0.7)	2.84a (0.08)				
	soluble	yes	ND	ND	ND	ND				
	insoluble	yes	85.3b (2.4)	93.1a,b (1.9)	101.4a (1.9)	1.95b (0.08)				

[a] Melting temperature and enthalpies of complexes scanned from 25 to 180 °C at 10 °C/min, immediately cooled from 180 to 25 °C at 40 °C/min and rescanned from 25 to 180 °C at 10 °C/min. Melting temperatures: Onset, T_o; Peak, T_p; Conclusion, T_c; and Enthalpy, ΔH.
[b] Mean (standard deviation) of at least two measurements. Means in a column not sharing the same letter are significantly different based on Tukey's honestly significant difference test ($p < 0.05$).
[c] ND, not detected

Table 3. Melting Temperatures and Enthalpies[a] of Recovered Soluble and Insoluble fractions of Unacetylated and Acetylated Potato Starch-Oleic Acid Complexes[b]. (Cont.)

starch	type of complex	β-amylase treatment	type I complex peak				type II complex peak			
			T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)
high acetylated	soluble	no	79.8c	86.1c	99.9a	1.86b				
			(1.5)	(0.8)	(0.4)	(0.16)				
	insoluble	no	80.7c	93.3a,b	100.9a	1.43c				
			(0.8)	(0.7)	(0.7)	(0.04)				
	soluble	yes	ND	ND	ND	ND				
	insoluble	yes	80.7c	91.6b	98.6a	1.73b,c				
			(0.1)	(0.2)	(2.0)	(0.06)				

[a] Melting temperature and enthalpies of complexes scanned from 25 to 180 °C at 10 °C/min, immediately cooled from 180 to 25 °C at 40 °C/min and rescanned from 25 to 180 °C at 10 °C/min. Melting temperatures: Onset, T_o; Peak, T_p; Conclusion, T_c; and Enthalpy, ΔH.
[b]Mean (standard deviation) of at least two measurements. Means in a column not sharing the same letter are significantly different based on Tukey's honestly significant difference test ($p < 0.05$).
[c]ND, not detected

The additional β-amylase treatment of unacetylated starches had no significant effect on the T_p and the enthalpy of the soluble complexes, but it decreased that of the insoluble complexes slightly, indicating that the β-amylase treatment resulted in starch chains that formed insoluble complexes with a lower stability and required a lower energy to dissociate.

The introduction of acetyl groups to starch chains significantly decreased the T_o and T_p for all complexes. These findings are in agreement with previous works,[22-23,40] in which acetylation of starch was reported to decrease the complexing ability and dissociation temperature of amylose-lipid complexes, although the complexes referred to in these studies were only the insoluble complexes. In addition, acetylated starch complexes displayed only the type I complex melting peak, supporting the previous study that the presence of the acetyl groups on starch may hinder the aggregation of the starch-oleic acid complexes.[23] However, there was no clear trend in melting enthalpy in terms of the β-amylase treatment. The enthalpy values also did not correlate with the amounts of complexed oleic acid as measured by GC (Table 2). This suggests that the enthalpy values might not necessarily represent the amount of oleic acid included in the starch complexes but might indicate the energy required to melt the starch-oleic acid complex due to the degree of order or level of organization existing within the complexes.

Although no melting endotherm was observed for the soluble complexes from the low acetylated without or with the β-amylase treatment and the high acetylated β-amylase treated starch, the presence of the starch-oleic acid complex was supported by the recovered oleic acid from the GC (Table 2) as well as by the presence of the V-type pattern displayed in the XRD results (Figure 5C,D&F). An opposite trend was observed in the soluble complexes of the low acetylated-only starch from the previous study with stearic acid,[23] in which the presence of the starch-stearic acid complex was not observed in the XRD and DSC analyses, but stearic acid was

recovered from the complexes as measured by GC. These results support the hypothesis that acetylated starch complexes may exist in different polymorphic arrangements[21] because the presence of acetyl groups on starch rendered them more soluble and affected their structural characterization by DSC. Nevertheless, there may be other factors causing the absence of the melting endotherm in some of the soluble starch-oleic acid complexes in the present study. Therefore, further investigations such as different chemical modifications or analytical techniques such as FTIR may provide a better understanding of the various types of polymorphs that can be generated during the complexation of acetylated starch and oleic acid and their structural characteristics.

CONCLUSIONS

In conclusion, the present study demonstrates that acetylation of starch alone can enhance the formation of both soluble and insoluble starch-oleic acid complexes compared with its unacetylated counterpart. Overall, high acetylated-only starch and low acetylated β-amylase treated starch resulted in the highest complex formation with oleic acid for the soluble and insoluble complexes, respectively. All acetylated soluble and insoluble complexes displayed the V-type X-ray diffraction pattern regardless of type of modification. The melting temperatures were reduced by acetylation for all complexes, however little correlation was found amongst the melting enthalpy values, X-ray intensity, and of the amount of either soluble or insoluble complex. The amount of complexed oleic acid in the present work was consistently lower than the amount of complexed stearic acid in the previous study for all treatments, confirming that the cis-configuration in fatty acids is not favored to form stable soluble or insoluble complexes and longer starch chains were preferred for complexation with oleic than with stearic acid.

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APPENDIX

Authorship Statement for Chapter 4

I verify that Emily Arijaje is the first author and completed over 51% of the research work published in the following article that is included in her dissertation: Effects of Chemical and Enzymatic Modifications on Starch-Oleic Acid Complex Formation.

Dr. Ya-Jane Wang

VII. Chapter 5 : Effects of Chemical and Enzymatic Modifications on Starch-Linoleic Acid Complex Formation

ABSTRACT

Acetylation has been used to reduce retrogradation and to improve solubility of starch. This study investigated the complexation yield and physicochemical properties of soluble and insoluble starch complexes with linoleic acid when acetylation and a β-amylase treatment were applied to debranched potato starch. The degree of acetylation was generally higher in the soluble complexes than in the insoluble ones. The insoluble complexes from the acetylated starch displayed the V-type pattern, whereas, the soluble complexes displayed a mixture of either the A-and V-type or the B-and V-type pattern. Acetylation decreased onset and peak melting temperatures for insoluble complexes, but the soluble complexes displayed no melting endotherm. Low acetylated starch resulted in the highest amounts of complexed linoleic acid in the insoluble complexes. Acetylation substantially increased the amount of complexed linoleic acid in the insoluble complexes, but had no positive effect on the formation of the soluble complexes.

KEYWORDS: acetylation, starch inclusion complex, linoleic acid, β-amylase, soluble complex, insoluble complex

INTRODUCTION

Inclusion complexes using starch as the complexing agent have been extensively studied.[1-8] Starch is mainly composed of two components, an essentially linear amylose molecule and a highly branched amylopectin molecule. Both amylose and amylopectin are polymers of glucose, and the linear portion of the chains may form a helical structure with a hydrophobic cavity that can include various hydrophobic ligands such as iodine,[9,10] alcohols,[11] lipids,[1,3,12-13] flavors,[14] and drugs.[6,13] When included in the starch helical cavity, the molecules are stabilized and protected from oxidation,[15] enzyme hydrolysis,[16] and high temperature.[17] Nevertheless, starch inclusion complexes are usually crystalline in nature and become insoluble in aqueous solutions.[18]

The complexation of starch and fatty acids have been reported and are influenced by many factors, such as starch chain length,[19,20] incubation temperature[17,21,22] and incubation pH.[23,24] The thermal stability of starch-fatty acid complexes increases with an increase in fatty acid chain length and decreases with an increase in fatty acid unsaturation.[25,26] Additionally, saturated fatty acids have been reported to form more stable complexes with starch compared with unsaturated fatty acids or mono or di-acylglycerols.[26-28] Most studies on starch inclusion complexes focused mainly on the formation of insoluble complexes, and only few studies have investigated soluble starch complexes.[29-31]

Recently, Arijaje et al.[30] and Arijaje and Wang[31] demonstrated that the formation of soluble complexes between starch and stearic (C18:0) and oleic acid (C18:1) could be significantly increased when starch was acetylated and debranched. The acetyl groups hindered starch retrogradation and encouraged its complexing with fatty acids. Low acetylated debranched starch with a degree of substitution (DS) ~ 0.04 increased the amount of complexed stearic and

oleic acid in both soluble and insoluble complexes. High acetylated starch (DS 0.08 – 0.09) also increased the amount of complexed stearic and oleic acid in the soluble complexes, but decreased the yield and complexed stearic and oleic acid in the insoluble complexes when compared with low acetylated starch. Stearic acid was complexed to a greater extent than oleic acid because it is saturated and relatively less soluble in water.[23] When a β-amylase treatment was incorporated with unacetylated and low acetylated debranched starch, the amount of complexed stearic and oleic acid generally increased in both soluble insoluble complexes. However when the β-amylase treatment was combined with high acetylated debranched starch, there was no consistent trend for either stearic or oleic acid.

This work continued our two previous studies[30,31] to investigate the impacts of fatty acid structure on the formation and properties of inclusion complexes with modified starch. The kinked structure in linoleic acid (C18:2) has been reported to present steric hindrance in the native starch helix, leading to only partial inclusion.[32] We hypothesized that modification of starch by chemical (acetylation) and enzymatic (isoamylase and/or β-amylase) methods could improve its complexation with linoleic acid.

MATERIALS AND METHODS

Materials. Potato starch was obtained from Penford Food Ingredients (Centennial, CO, USA) and used without further treatment. Isoamylase from *Pseudomonas sp* (specific activity 280 units/mg protein), pullulanase from *Klebsiella planticola* (specific activity 34 units/mg protein) and β-amylase from *Bacillus cereus* (specific activity 2660 units/mg protein) were purchased from Megazyme International Ireland Ltd. (Wicklow, Ireland). Linoleic (*cis*-9, *cis*-12-

Octadecadienoic) acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of ACS grade.

Acetylation of Starch. Starch was acetylated as previously described by Wang and Wang.[33] The acetylation level of starches were determined according to the method of McComb and McCready[34] and the degree of substitution (DS) were determined according to Wurzburg.[35]

Enzymatic Modification of Starch

Debranching. Starch was debranched as previously described by Arijaje et al.[30] Potato starch (3.75%, w/v, starch db) was gelatinized in a boiling water bath for 1 h with constant stirring. Then the temperature of the solution was equilibrated to 45 °C and the pH adjusted to 5.0 with 0.5 M HCl. To the starch solution, isoamylase and pullulanase (1.33% v/w starch db) each was added, and incubated at 50 °C with constant stirring for 48 h. The starch was recovered with 4-fold volume of pure ethanol, centrifuged at 7000*g* for 10 min, dried at 40 °C for 48 h, and ground into powder using a UDY cyclone mill (UDY Corp., Ft. Collins, CO, USA) fitted with a 0.5-mm screen.

β-Amylase Treatment. A portion of the debranched starch was subjected to an additional β-amylase hydrolysis to reduce the degree of polymerization (DP). After the debranching for 48 h, the starch slurry pH was adjusted to 6.5 with 0.5 M NaOH, and incubated with 0.5% (v/w starch db) β-amylase at 40 °C for 4 h. The enzyme reaction was terminated by boiling for 15 min. The β-amylase-treated starch was recovered as previously described.

Characterization of Starch Structure. The DPs of debranched unacetylated and acetylated starch without or with β-amylase treatment were determined by recovering starches after complexation with linoleic acid. The recovered starches were characterized using a high-performance size-exclusion chromatography (HPSEC) system (Waters Corp., Milford, MA,

USA). Starch (10 mg) was dissolved in 5 mL of 90% DMSO, boiled for 1 h, and filtered through a 5.0-μm filter prior to injection into the HPSEC system. The HPSEC system consisted of a guard column (OHpak SB-G, 6.0 × 500 (mm) i.d. × length), two Shodex columns (OHpak KB-804 and KB-802, both 8.0 × 300 (mm) i.d. × length), a 200 μL injector valve (model 7725i, Rheodyne, Cotati, CA, USA), an inline degasser, a model 515 HPLC pump, and a model 2414 refractive index detector. The mobile phase of 0.1 M sodium nitrate with 0.02% sodium azide was eluted at a flow rate of 0.6 mL/min. The temperature of column was maintained at 60ºC and the detector at 40ºC. Dextran standards of molecular weight of 5,200, 11,600, 23,800, 48,600, 273,000 and 410,000 g/mole from Waters Corp. (Milford, MA, USA) and 1,050,000 g/mole from Sigma Aldrich (St. Louis, MO, USA) were used to establish the calibration curve.

The amylopectin chain length distributions were characterized by high-performance anion-exchange chromatography equipped with pulsed-amperometric detection (HPAEC-PAD) according to the method of Wong and Jane.[36] The chains were divided into DP ranges and classified as follows: A chains (DP 6-12), B1 chains (DP 13-24), B2 chains (DP 25-36), and B3+ chains (DP 37+).[37] The average chain length was calculated as the cumulative sum of the product of DP and percentage relative areas for all the identified peaks.

Complexation of Starch and Linoleic Acid. The starch solution (3.75% w/v), debranched or debranched and β-amylase treated, was adjusted to pH 7.0 and preheated to 80 °C, and mixed with 1 g of linoleic acid that was dissolved in warm 95% ethanol. The mixture was maintained at 80 °C for 30 min with continuous stirring to allow complexation, and then the temperature was maintained at 45 °C overnight with continuous stirring. The resulting starch-linoleic acid mixture was centrifuged at 7000*g* for 10 min, from which the precipitate, "*insoluble complex*", was obtained, whereas, the "*soluble complex"* was recovered by precipitating the supernatant with 4-

fold volume of pure ethanol. Any uncomplexed linoleic acid was removed from both the *insoluble* and *soluble* complexes by rotating complexes in excess 95% ethanol using a labquake shaker rotisserie (Barnstead/Thermolyne, Dubuque, IA, USA) at room temperature for 2 h, centrifuged at 7000g for 10 min, dried at 40 °C for 48 h, milled using a mortar and pestle, sieved through a 250-mm sieve, and stored for further analysis.

Hydrolysis of Complexes and Linoleic Acid Analysis. Hydrolysis of complexes was carried out as described by Arijaje et al. (2014). Soluble or insoluble complex (100 mg) was added with 10 mL of 1 M HCl and heated with continuous stirring in a boiling water bath for 1 h. After the complex mixture was cooled to room temperature, 5 mL hexane was added, and the solution was rotated on the rotary shaker for 1 h. The hexane layer with the extracted linoleic acid was recovered, and the extraction was repeated with another 5 mL hexane for 1 h. To the recovered hexane phase, boron trifluoride methanol was added to convert linoleic acid to linoleic acid methyl esters. An internal standard of methyl heptadecanoate (~1 mg) was subsequently added to all complexes. The linoleic acid methyl ester was injected into a gas chromatographer (GC) (GC-2010, Shimadzu, Kyoto, Japan) equipped with a BP 21 capillary column (30 m × 0.25 mm i.d.; SGE Inc., Austin, TX) with a flame ionization detector (FID), and responses were collected by Shimadzu GCsolution Workstation 2.3 (Kyoto, Japan). The injection port and detector temperatures were set at 220 °C and 230 °C, respectively. The column oven temperature was equilibrated at 100 °C for 1 min, ramped up at 15 °C /min to 160 °C, again ramped up at 5 °C /min to 200 °C and maintained at 200 °C for 10 min. The flow rate of the carrier gas (helium) was 30 mL/min. The concentration of linoleic acid was determined from a standard curve prepared by using methylated linoleic acid solution (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL) containing the internal standard of methyl heptadecanoate (0.5 mg/mL).

Physicochemical Properties. X-ray diffraction (XRD) patterns of complexes were obtained using a diffractometer (PW1830 MPD, Philips, Almelo, The Netherlands). The XRD diffractograms were collected with the generator voltage set at 45 kV and the current set at 40 mA. Samples were scanned over the 2θ angle from 5° to 35° in 0.02° steps at 1 s per step.

The thermal properties of complexes were analyzed with a differential scanning calorimeter (DSC, Pyris-Diamond, PerkinElmer, Shelton, CT, USA). Complexes (approximately 8 mg) were weighed into stainless steel pans, 16 μL of distilled water was added with the aid of a microsyringe, and the pans were hermetically sealed. The samples were equilibrated for 24h at room temperature before scanning and were scanned from 25 to 180 °C at a rate of 10 °C/min, and to confirm the formation of the starch-fatty acid complex, the complexes were immediately cooled from 180 to 25 °C at a rate of 40 °C/min and rescanned from 25 to 180 °C at a rate of 10 °C/min. The onset melting temperature (T_o), peak melting temperature (T_p), conclusion melting temperature (T_c) and enthalpy (ΔH) of the endotherms of the rescanned complexes were calculated using the Pyris data analysis software.

Statistical Analysis. The JMP software (SAS Institute Inc., Cary, NC, USA) was used to analyze the statistical data that were conducted in replication, and the means were compared using Tukey's honestly significant differences (HSD) test.

RESULTS AND DISCUSSION

Degree of Substitution. The DS of acetylated starch and starch complexes are presented in Table 1. The low and high acetylated starches had a DS of 0.045 and 0.091, respectively, prior to complexing with linoleic acid. The DS of acetylation was higher in the soluble complexes than in the insoluble ones within the same treatment, except for the low acetylated-only starch complex,

in which the DS in the soluble complex (0.048) was lower than in the insoluble one (0.063). The present result follows the trend that soluble complexes with linoleic acid would form at a lower DS (0.048), which was similar to oleic acid (DS 0.045),[31] than with stearic acid (DS 0.063), because its double bond that increases its hydrophilicity.[23] When the β-amylase treatment was included, the DS of acetylation increased in all soluble and insoluble complexes except for the low acetylated-only insoluble complex. This agrees with previous works[30,31] that acetyl groups were preserved during the β-amylase hydrolysis of acetylated starch.

Table 1. Degree of Substitution (DS) of Acetylated Starches and Starch-Linoleic Acid Complexes[a].

starch	**β-amylase treatment**	**type of complex**	**DS**
low acetylated starch	N/A	N/A[b]	0.045±0.001f
low acetylated starch complex	no	soluble	0.048±0.001e
		insoluble	0.063±0.001d
	yes	soluble	0.092±0.000b
		insoluble	0.046±0.000f
high acetylated starch	N/A	N/A	0.091±0.001b
high acetylated starch complex	no	soluble	0.090±0.000b
		insoluble	0.037±0.001g
	yes	soluble	0.140±0.000a
		insoluble	0.076±0.000c

[a]At least replicate samples were prepared for each complex. Data of two measurements with standard deviation. Means in a column not sharing the same letter are significantly different based on Tukey's honestly significant difference test ($p < 0.05$).
[b]N/A, not applicable.

Complex Recovery and Complexation Yield. Complex recovery was the recovered complex weight over the initial material weight (Table 2). Total recovery including both soluble and insoluble complexes for all unacetylated and acetylated complexes was between 0.90-0.95 g/g, except the unacetylated β-amylase-treated complex with a total recovery of 0.87 g/g.

Table 2. Complex Recovery and Linoleic Acid Content Recovered from All Complexes[a].

starch	type of complex	β-amylase treatment	recovery (g/g)	linoleic acid in complex (mg/g)
Unacetylated	soluble	no	0.29±0.02f,g	1.0±0.0h
	insoluble	no	0.65±0.00b,c	1.2±0.1g
	soluble	yes	0.11±0.01h	1.1±0.0g,h
	insoluble	yes	0.76±0.01a	2.1±0.1e
low acetylated	soluble	no	0.57±0.03c,d	1.5±0.0f
	insoluble	no	0.34±0.06f	26.7±0.4b
	soluble	yes	0.47±0.02e	2.3±0.0e
	insoluble	yes	0.48±0.00d,e	54.4±1.6a
high acetylated	soluble	no	0.74±0.02a,b	1.2±0.0g
	insoluble	no	0.20±0.03g,h	19.2±0.2c
	soluble	yes	0.65±0.02b,c	1.1±0.0g,h
	insoluble	yes	0.25±0.01f,g	12.5±0.4d

[a]Data of at least two measurements with standard deviation. Means in a column not sharing the same letter are significantly different based on Tukey's honestly significant difference test ($p < 0.05$).

The total recovery is similar to those with oleic acid[31] and supports previous findings that modified starch only changed the proportion of soluble and insoluble complexes and had little impact on the total complex recovery. Similar to the previous works,[30,31] for the unacetylated

starches, the recovery of the insoluble complexes was higher than that of the soluble complexes and increased with the β-amylase treatment. The recovery for soluble complexes increased with increasing acetylation level but decreased when the β-amylase treatment was combined for the same treatment, and the opposite was noted for the insoluble complexes.

The amount of linoleic acid recovered from unacetylated complexes was very low (1.0 - 2.1 mg/g) compared with stearic acid (15.7 – 63.1 mg/g) and oleic acid (3.0 – 10.9 mg/g) in previous studies. Acetylation of starch significantly increased the amount of complexed linoleic acid in the insoluble complexes because the re-association of starch was reduced and therefore complex formation was favored. Acetylation increased the amount of complexed linoleic acid in the soluble complexes, although overall complexation yield was still low. The presence of a high number of acetyl groups may create steric hindrance that reduced complex formation in the soluble complexes. When the β-amylase treatment was combined, the amount of complexed linoleic acid increased in both complexes for the low acetylated starch; for the high acetylated complexes, the amount of complexed linoleic acid remained unchanged in the soluble complexes but greatly decreased in the insoluble complexes. The low inclusion of linoleic acid for the high acetylated β-amylase treated starches was attributed to a combination of shorter starch chains from the β-amylase hydrolysis and steric hindrance from the increased acetyl groups. The present results for the insoluble acetylated complexes are not consistent with results of Lui et al.,[38] who reported that acetylation of pea starch decreased its complexing ability with lauric acid (C12:0) and monopalmitin. The differences between the two studies may be due to the higher DS of acetyl groups (DS~0.1) in Lui et al.[38] compared with DS of 0.037 to 0.076 in the present study. The DS of acetylation ~0.1 in Lui et al.[38] may be too high to encourage proper complex formation because the previous and present studies also demonstrated that a high acetylation

level had a negative impact on starch complexation with fatty acids.[30,31] In addition, the different botanical source and treatment of starch used in Lui et al.[38] (native pea starch) compared with debranched starch in this study may also contribute to the inconsistency.

Low acetylated β-amylase treated starch complexed the highest amount of linoleic acid (54.4 mg/g) in the insoluble complexes, which also complexed the highest amount of stearic acid (123.1 mg/g) and oleic acid (42.9 mg/g).[30,31] The results suggest that a combination of low acetylation and β-amylase treatment improves starch complexing with fatty acids to form insoluble complexes. Acetylation alone or in combination with the β-amylase treatment did not greatly improve soluble starch complex formation with linoleic acid, although it increased soluble complexes with stearic and oleic acid considerably. This low complexation yield of the soluble complexes with linoleic acid is attributed to its kinked structure and greater affinity for the complexing solvent compared with stearic and oleic acid because Hahn and Hood[23] reported that a compound with a greater affinity for the complexing solvent than the starch helix may remain more in the unbound state than as part of the starch complex.

Molecular Size Distribution. The standard curve used to estimate the degree of polymerization of debranched starch complexes without or with the β-amylase treatment is displayed in Figure 1. The molecular size distributions of starch chains recovered from both soluble and insoluble complexes of all treatments are presented in Figure 2. The soluble and insoluble complexes displayed a peak at a peak retention time of 18.2-18.7 min, indicating the presence of amylose with a DP range of approximately 4000-7000, which was reported to be too long to participate in complex formation.[39] The proportion of this amylose fraction increased with acetylation, similar to previous works,[30-31] because of its increased hydrodynamic volumes from the presence of acetyl groups, and the increase was greater in the insoluble complexes than in the soluble ones.

The proportion of the amylose fraction was further increased when acetylated starches received the additional β-amylase treatment, presumably because of a further increase in hydrodynamic volume of starches and the hydrolysis of amylopectin short chains. The major peaks observed in the soluble complexes were composed of amylopectin branched chains at a peak retention time between 23.8 and 24.8 min, which corresponded to DP 44 and 17, respectively, and this peak retention time also decreased with increasing acetylation level. The insoluble complex showed a narrower peak retention time between 23.3 and 24.0 min, which corresponded to DP 69 and 36, respectively, indicating that amylopectin chains participated in complex formation with linoleic acid.

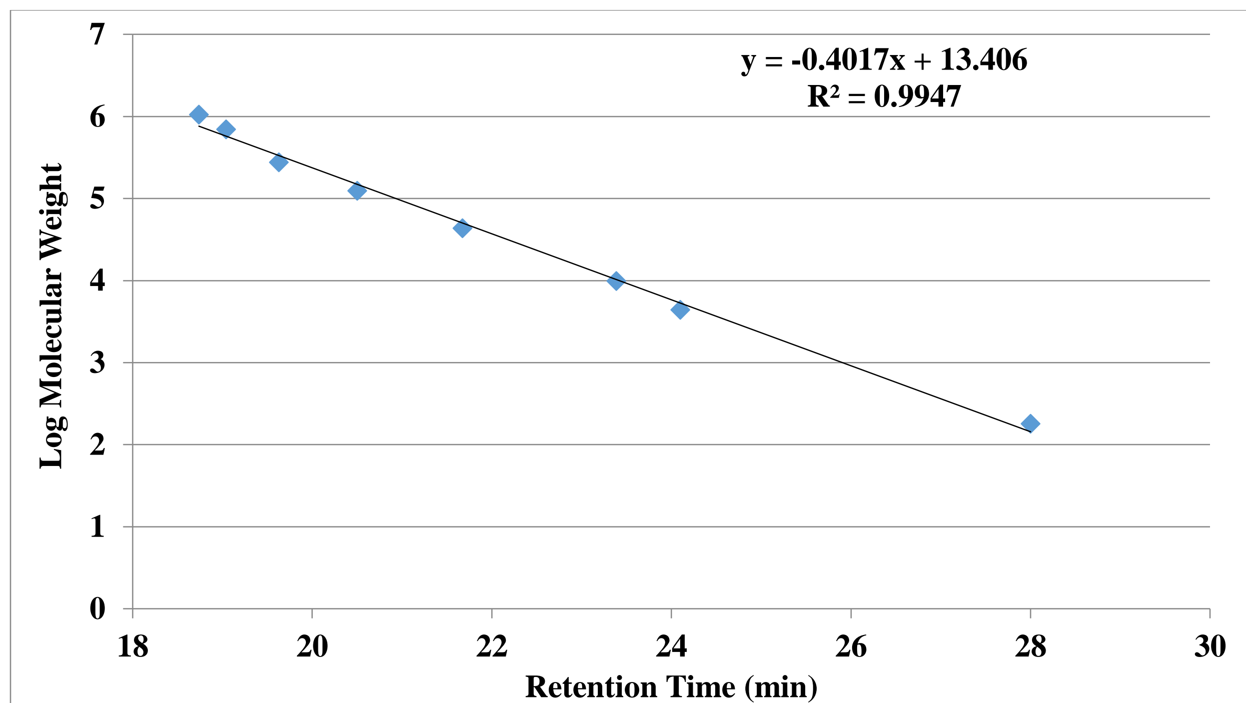

Figure 1. Standard curve used to estimate the degree of polymerization of debranched starch complexes without or with the β-amylase treatment.

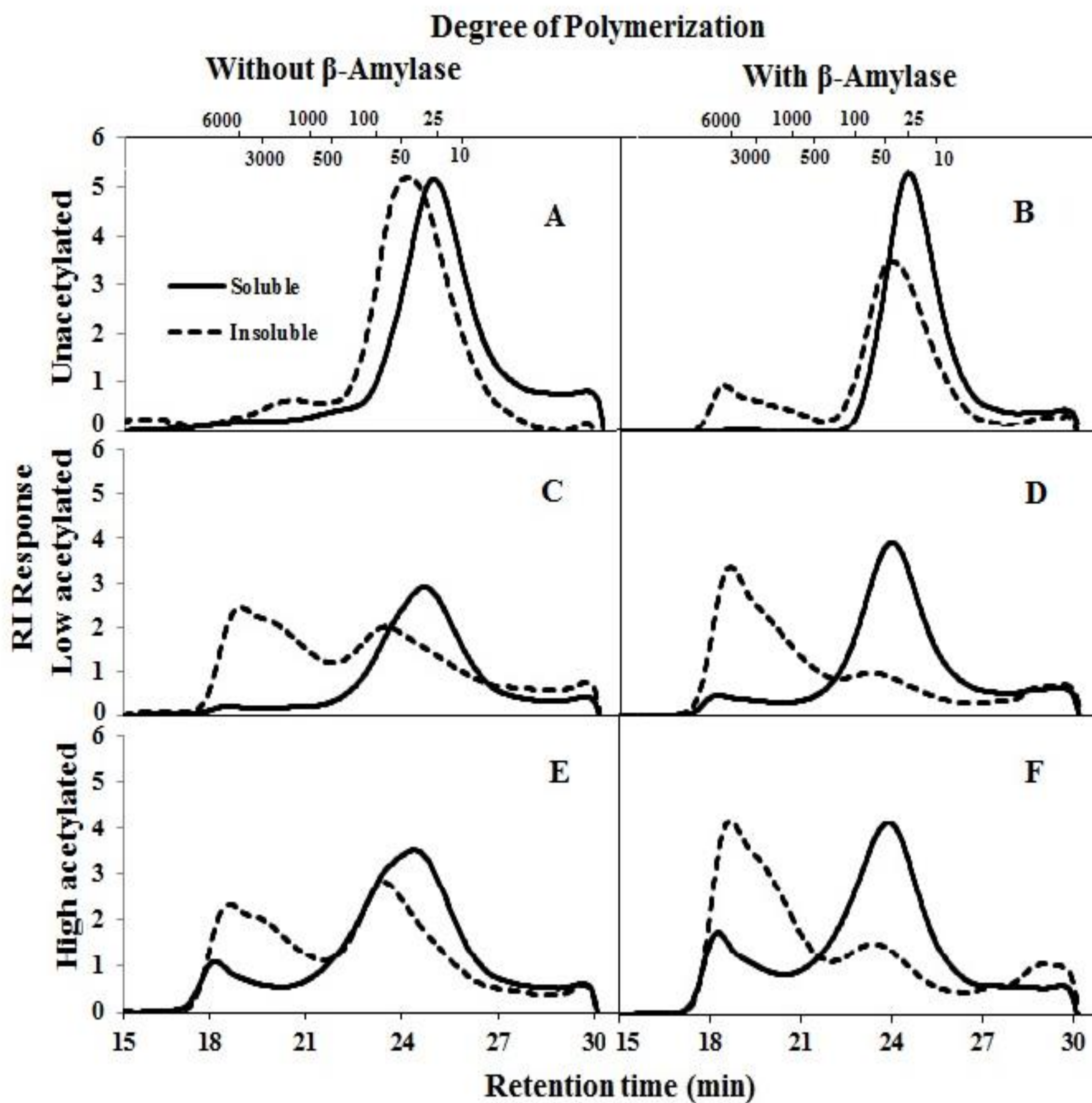

Figure 2. Normalized size-exclusion chromatograms of recovered soluble and insoluble starch complexes from unacetylated and acetylated starches after complexing with linoleic acid: (A) starch; (B) β-amylase-treated starch; (C) low acetylated starch; (D) low acetylated β-amylase-treated starch; (E) high acetylated starch; (F) high acetylated β-amylase-treated starch.

The peak retention time of the soluble complexes was at a lower DP range and the proportion of longer starch chains was greater in the insoluble complexes than in the soluble complexes for the same treatment, supporting that longer chains were required to form the insoluble complexes.

When the β-amylase treatment was included, the main peak became narrower and shifted to a higher DP range, presumably because the shorter chains were hydrolyzed by β-amylase and the DS of acetylation became higher. The low acetylated β-amylase treated starch complexed the highest amount of linoleic acid for the insoluble complexes and had a peak DP 72, which was similar to the result by Xu et al.[40], in which synthesized amylose with a DP 62 was effective in forming complexes with linoleic and linolenic acid. The present results agree with previous works[30-31] that starch chains with DPs ~50-80 and a low acetylation degree (~0.034-0.046) improved the formation of insoluble complexes with stearic and oleic acids. For the soluble complexes, since overall complexation yield was very low compared with the insoluble complexes, the present results support our idea that there is an optimum combination of acetylation and starch chain length that will encourage complexation. And in the case of the soluble complexes from the starch-linoleic acid complex, the right starch chain length and/or in combination with acetylation to encourage complexation was not achieved.

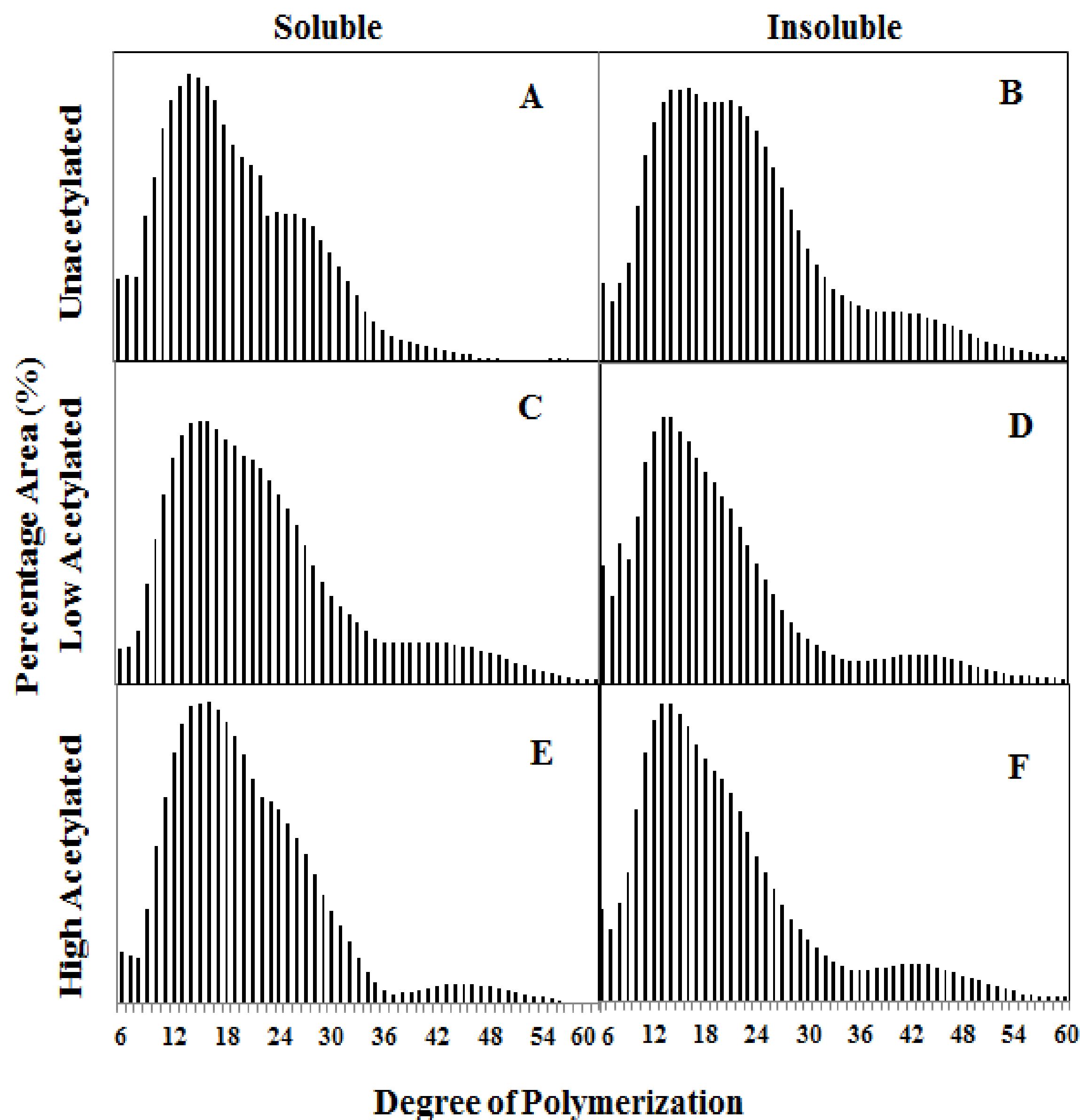

Figure 3. Normalized chain-length distributions of recovered soluble and insoluble starch complexes from unacetylated and acetylated starches without the β-amylase treatment after complexing with linoleic acid using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD): (A) starch; (B) β-amylase-treated starch; (C) low acetylated starch; (D) low acetylated β-amylase-treated starch; (E) high acetylated starch; (F) high acetylated β-amylase-treated starch.

Soluble starches demonstrated a lower average DP range compared with the insoluble complexes as shown by the HPSEC chromatogram (Figure 2), therefore starch chains may not have favored complexation with linoleic acid. In addition, the presence of high acetyl group content of the short chained soluble starch which ranged from 0.048-0.140 may have introduced steric hindrance during complexation with the kinked linoleic acid.

The chain length distributions of the recovered amylopectin chains without and with the β-amylase treatment are displayed in Figures 3 and 4, respectively. For the debranched-only starches, most main peaks had a peak DP 13-16 and a minor peak of DP 40-44. Godet et al.,[12] had reported that starch chains with DP < 20 were too short to complex with lipids.

When the additional β-amylase treatment was included, the proportion of chains with DP 20-60 increased, while that of chains with DP 6-20 decreased noticeably. This agrees with the HPSEC results that the additional β-amylase treatment created more long chains with favorable lengths to complex linoleic acid. Both HPSEC and amylopectin chain length distribution results are supported by the higher amounts of linoleic acid in the insoluble complexes of unacetylated and low acetylated starches that received the additional β-amylase treatment (Table 2). Moreover for the β-amylase treatment of high acetylated starches, the amount of complexed linoleic acid decreased slightly, indicating that there exists an optimum combination of DS of acetylation and starch chain length to encourage complex formation.

For the soluble complexes from all treatments, complexation was very low, presumably because shorter chains formed these soluble complexes,[30] therefore, the proportion of starch chains with DP > 20 in the soluble starch chains in the present work were lower than that of the insoluble ones, supporting the HPSEC results. These observations also agree with our earlier explanation that the starch chains of the soluble complexes might not be sufficiently long enough

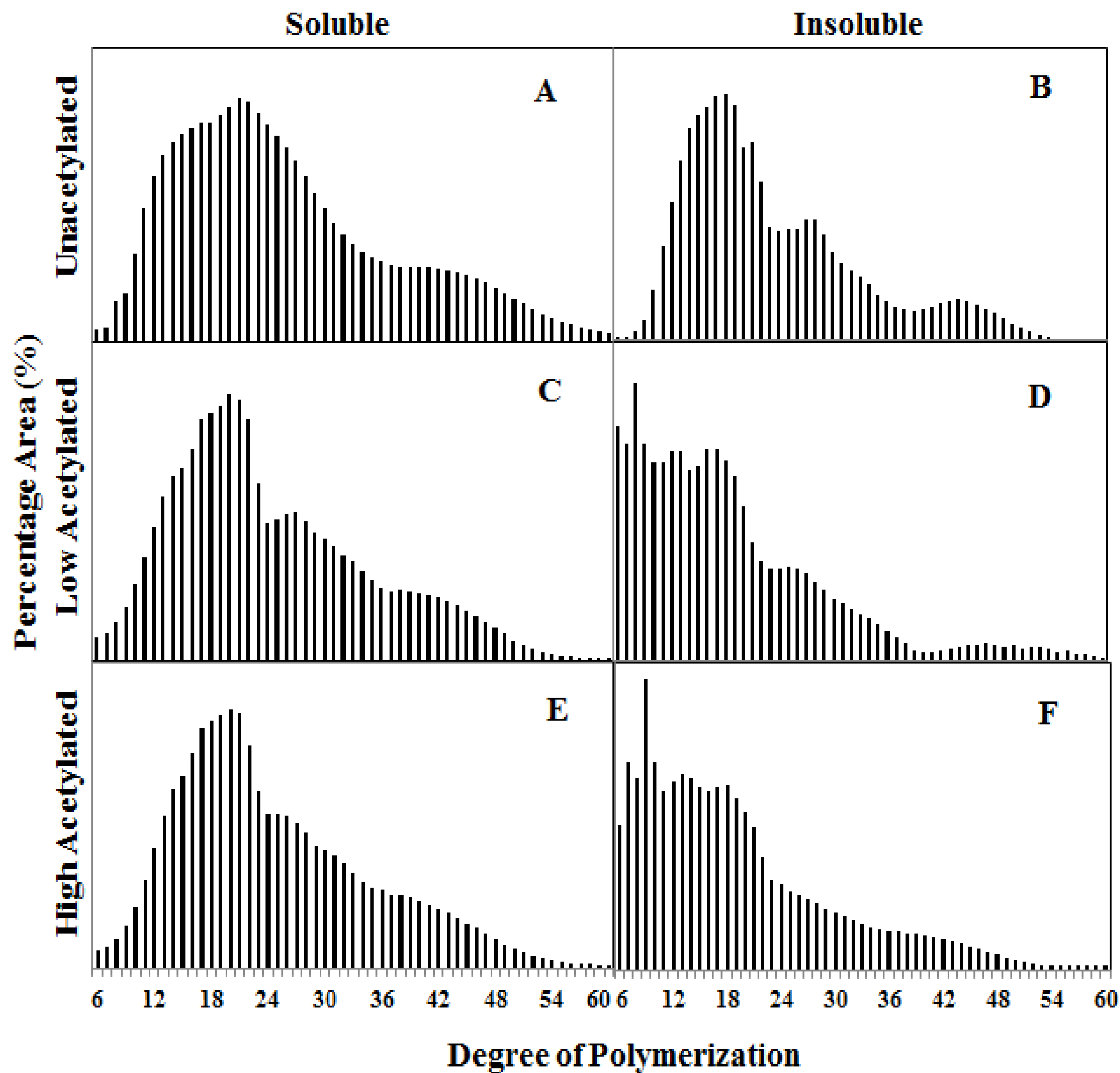

Figure 4. Normalized chain-length distributions of recovered soluble and insoluble starch complexes from unacetylated and acetylated starches with the β-amylase treatment after complexing with linoleic acid using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD): (A) starch; (B) β-amylase-treated starch; (C) low acetylated starch; (D) low acetylated β-amylase-treated starch; (E) high acetylated starch; (F) high acetylated β-amylase-treated starch.

to participate in the formation of stable complexes with linoleic acid because the kinked structure of linoleic acid may have found it difficult to fit into the short chained helices.[23] Therefore, acetylation without or with the β-amylase treatment on starch had little impact in improving soluble complex formation with linoleic acid.

Characterization of Starch-Linoleic Acid Complexes

X-ray Diffraction Pattern. All unacetylated complexes displayed the B-type X-ray diffraction pattern with peaks at 2θ = 14.2º, 17.2º, 19.5 º, 22º and 24º, except that the unacetylated debranched-only soluble complex displayed the A-type pattern with peaks at 2θ = 10º, 11.4º, 15.2º, 17.1º, 18.2 º, and 23º (Figure 5A,B). The characteristic V-type pattern by starch-fatty acid complexes was not observed in all unacetylated starches except for the peak occurring at 2θ = 20º, which support the presence of linoleic acid as measured by GC (Table 2).

Acetylation of starch increased the peak intensities and the characteristic V-type pattern of starch-fatty acid complexes[12,41,42] with peaks at 2θ = 7.6º, 12.9º and 20º in all insoluble complexes (Figure 5C-F). The peak intensities of the insoluble complexes from linoleic acid were much higher than those from stearic or oleic acid for the same treatment.[30-31] The interaction between acetylated starches and linoleic acid may result in insoluble complexes with a more crystalline structure or a different type of polymorphs due to the presence of acetyl groups when compared with the unacetylated starches. The intensity of the V-type pattern increased with the additional β-amylase treatment but did not change significantly with an increase in DS of acetylation. The trend of the increasing V-type peak intensity was also observed for the stearic and oleic acid in the previous works.[30,31] This indicates that the crystallinity of the V-type pattern of complexes became more evident as a result of the

hydrolysis of the of starch chains that were not involved in complex formation by β-amylase, therefore the V-type pattern became more dominant.

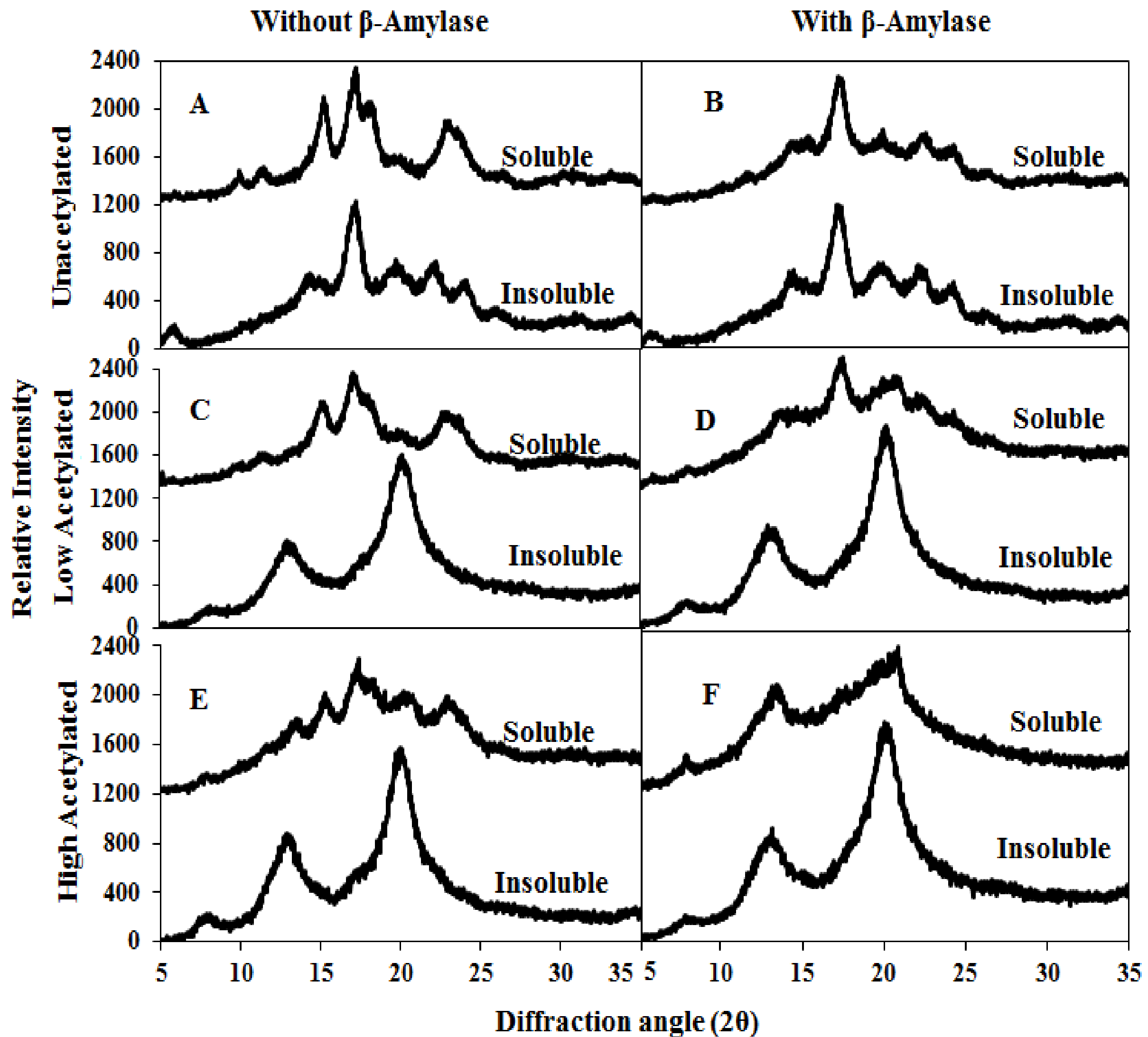

Figure 5. Normalized X-ray diffractograms of recovered soluble and insoluble starch complexes from unacetylated and acetylated starches after complexing with linoleic acid: (A) starch; (B) β-amylase-treated starch; (C) low acetylated starch; (D) low acetylated β-amylase-treated starch; (E) high acetylated starch; (F) high acetylated β-amylase-treated starch.

The soluble complexes from the low and high acetylated-only starch mainly displayed the mixed A- and B-type X-ray patterns similar to as their unacetylated counterparts, indicating that the acetylation did not improve complexation (Figure 5C,E). When the β-amylase treatment was included, the V-type pattern became more visible in the soluble complex of low-acetylated starch (Figure 5D) and was evident in the high acetylated starch complex (Figure 5F). However, the V-type pattern displayed by the soluble complex of the high acetylated and β-amylase treated starch showed a shoulder at the lower diffraction angle of all three peaks. This clearly signifies that a different structural arrangement is present in this specific sample, which was not observed in the other samples in the present and previous works.

Despite the V-type pattern observed in the soluble complexes from high acetylated β-amylase treated starch, the amount of linoleic acid recovered was very low, supporting Arijaje & Wang[31] that the intensity of the V-type pattern was not necessarily correlated with the quantity of included molecule but may more reflect the level of organizational arrangement of the complexes formed. Because very little information exists in literature for the XRD patterns that are exhibited by chemically and enzymatically modified starch complexes, more research is needed to understand the structures of these soluble complexes.

Melting Properties by Differential Scanning Calorimetry. The unacetylated starch complexes displayed an onset melting temperature except for the debranched-only insoluble complex at with (T_o) 79.2 – 89.0 °C, and peak melting transition (T_p) 83.9 - 95.6 °C, which denotes the I complex that is known to consist of randomly distributed helical segments[43] with a melting temperature around ~95 °C.[22,27]

Table 3. Melting Temperatures and Enthalpies[a] of Recovered Soluble and Insoluble of Unacetylated and Acetylated Starch-Linoleic Acid Complexes[b].

Starch	type of complex	β-amylase treatment	Starch-linoleic acid complex			
			T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)
Unacetylated	soluble	no	89.0a (0.4)	95.0a (0.2)	101.1a (0.3)	1.62b,c (0.13)
	insoluble	no	ND	ND	ND	ND
	soluble	yes	88.8a (0.2)	95.6a (0.5)	103.1a (0.6)	3.27a (0.14)
	insoluble	yes	79.2b (0.9)	83.9b (0.7)	87.8c,d (0.4)	0.35e (0.10)
low acetylated	soluble	no	ND[c]	ND	ND	ND
	insoluble	no	72.6c (0.7)	84.1c (0.4)	90.3b,c (0.9)	1.39c,d (0.15)
	soluble	yes	ND	ND	ND	ND
	insoluble	yes	72.4c (0.5)	80.8c (0.6)	87.3d (1.1)	1.13d (0.16)
high acetylated	soluble	no	ND	ND	ND	ND
	insoluble	no	73.0c (1.2)	81.6c (0.4)	89.9b,c,d (0.7)	1.61b,c,d (0.04)
	soluble	yes	ND	ND	ND	ND
	insoluble	yes	70.7c (0.4)	83.6b (0.4)	91.2b (0.0)	1.96b (0.05)

[a]Melting temperature and enthalpy after complexes were scanned from 25 to 180 °C at 10 °C/min, cooled from 180 to 25 °C at 40 °C/min and rescanned from 25 to 180 °C at 10 °C/min. Melting temperatures: Onset, T_o; Peak, T_p; Conclusion, T_c; Enthalpy, ΔH.
[b]Mean (standard deviation) of at least two measurements. Means in a column not sharing the same letter are significantly different based on Tukey's honestly significant difference test ($p < 0.05$).
[c]ND, not detected

The type II complex consists of aggregates of type I complexes with well-defined crystalline structures and melts at ~110 °C.[43] The formation of the type I complex in the present study maybe be due to the kinked structure of linoleic acid which prevented the formation of more

crystalline complexes. The reason for the absence of the melting peak in the unacetylated insoluble complex is not clear.

All acetylated insoluble starch complexes displayed only the type I complexes with T_p between 80.8 to 84.1 °C. The T_p values reported in the present work were generally lower than those reported in the previous works[30-31] with stearic or oleic acid, but still represented the melting of the starch complex because the complexes were reheated and $T_p > 75$ °C had previously been reported to denote the melting temperature of amylose-lipid complex.[26,28] Acetylation decreased the T_o, T_p and enthalpy for most starches because the presence of the acetyl groups hindered the formation of more ordered starch complexes, thus reducing their melting temperatures. There was no further reduction in melting temperatures and enthalpy values with high acetylation for starch-linoleic complexes, which was different from starch complexes with stearic and oleic acids where high acetylation resulted in starch complexes of lower melting temperature and enthalpy than did low acetylation. It is possible that the kinked structure of linoleic acid itself destabilizes the starch helix. In addition, linoleic acid complexes displayed a lower T_o compared with stearic and oleic acid complexes, suggesting that the starch-linoleic acid complex had a lower degree of order or arrangement. This agrees with the findings by Karkalas et al.[22] (1995) and Zabar et al.[42] that an increase in the degree of unsaturation of fatty acids used in starch complexation leads to the production of ill-defined crystallites with low thermal stability and spatial localizability. There was no melting endotherm for the soluble complexes from the acetylated starches, which was also supported by the GC and XRD results that the acetylated starch soluble complexes included very small amounts of linoleic acid.

Overall, the enthalpy values were consistently lower in the acetylated complexes compared with their unacetylated counterparts, indicating that a lower energy was required to

disrupt the acetylated complexes[30] because of its improved solubility from the incorporation of the acetyl groups. The interference of re-associated amylose and amylopectin in these complexes was excluded as the complexes were rescanned immediately after prior heating and cooling. However, the enthalpy values were not consistent with the amounts of complexed linoleic acid for unacetylated and acetylated complexes (Table 2). Therefore the enthalpy values may represent the amount of energy required to disrupt and melt the complex, and may reflect the order existing within the complexes rather than the amount of linoleic acid in the complexes.

CONCLUSIONS

In conclusion, the present study showed that acetylation alone or combined with a β-amylase treatment can improve the amount of complexed linoleic acid in the insoluble complexes, but did not increase the soluble complexes of linoleic acid. The bent structure of the linoleic acid hindered soluble complex formation. Acetylation decreased the melting temperature and enthalpy of all starch-linoleic acid complexes. Low acetylation combined with the β-amylase treatment of starch resulted in the highest amount of complexed linoleic acid in the insoluble complexes. There was an optimum degree of acetylation and starch chain length for improving complex formation for the insoluble complexes. A combination of low acetylation and β-amylase treatment of starch can be exploited for the preparation of the insoluble starch-inclusion complexes with bioactive compounds, and this treatment combination may help improve the stability of these complexes and ultimately improve their bioavailability.

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Appendix

Authorship Statement for Chapter 5

I verify that Emily Arijaje is the first author and completed over 51% of the research work published in the following article that is included in her dissertation: Effects of Chemical and Enzymatic Modifications on Starch-Linoleic Acid Complex Formation

Dr. Ya-Jane Wang.

VIII. OVERALL CONCLUSIONS

Debranching of starch created more linear starch chains that could participate in complex formation for potato, common corn and Hylon VII starches, but the effects of an additional β-amylase treatment varied with different starches. ITC showed that debranched waxy maize starch and potato amylose bound to hexanoic acid through an exothermic cooperative event, with debranched waxy maize starch binding faster to hexanoic acid than potato amylose. Acetylation increased complex formation for both soluble and insoluble complexes by decreasing starch retrogradation and stabilizing the included fatty acids. Degree of acetylation was generally higher for the soluble complexes compared with the insoluble ones within the same treatment. A combination of low acetylation and the β-amylase treatment included the highest amounts of fatty acids (stearic, oleic and linoleic acids) for the insoluble complexes. High acetylated starch with and without the β-amylase treatment can be employed for the production of soluble complexes as this starch treatment included the highest amount of stearic acid and oleic acid, respectively. There was an optimum starch chain length and degree of acetylation required to encourage the formation of these complexes. Complex formation increased for all starches with an increase in the proportion of starch chains with DP 20 to 400, and starch chains with DP > 400 may also actively participate in complexation. The information from this study could provide insight into ways of increasing complexation yield, and into the creation of soluble complexes between starch and other insoluble bioactive compounds, which may lead to the increased digestibility and bioavailability of the included bioactive compound.