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Effects of Chemical and Enzymatic Modifications on Starch and Naringenin Complexation

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Effects of Chemical and Enzymatic Modifications on Starch and Naringenin Complexation

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ABSTRACT

Naringenin is a flavanone naturally present in grapefruit and tomato skin, which has been demonstrated to have health benefits. However, because of the low water solubility and bioavailability, naringenin applications are limited. Starch inclusion complexes have been shown to improve the solubility and bioavailability of poorly water soluble bioactive compounds. The present study aimed to prepare and characterize complexes of naringenin with starches, including potato starch and high amylose corn starch (Hylon VII), which were chemically (acetylation or hydroxypropylation) and enzymatically modified (debranched or debrahced/β-amylase treated). Soluble and insoluble complexes were recovered, and their physicochemical properties were characterized. The treatments did not affect overall recovery, but the introduction of acetyl and hydroxypropyl groups significantly increased the recovery of soluble complexes. Overall, acetylated starches exhibited greater complexation yields than hydroxypropylated counterparts; Hylon VII complexes comprised greater naringenin contents than potato starch complexes. The naringenin content generally was greater in insoluble complexes than in soluble complexes and increased when β-amylase treatment was incorporated. The X-ray diffraction patterns of both complexes revealed a mixture of amorphous and crystalline structure. FT-IR results confirmed the occurrence of molecular interaction between starch and naringenin in both complexes. Melting properties were significantly influenced by the type and degree of substitution. The present results demonstrate that the complexation of starch with naringenin can be improved by a combination of chemical and enzymatic modifications.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Starch is the main carbohydrate form in plants as an energy reserve. The characteristics and functionality of starch differ among botanical sources and are governed by its chemical composition and structure. Starch is composed of two different types of polymers, amylose and amylopectin, both having α-(1→4) linked anhydroglucose backbone but differing in their molecular sizes and branching nature. Amylose is an essentially linear molecule, while amylopectin is a highly branched molecule consisting of branches linked through α-(1→6) glucosidic linkages. In aqueous solutions, amylose mainly exists as random coils and tends to form double helices in order to maintain a favorable energy state. The formation of double helices requires the interaction and alignment of two molecules, but it could be interrupted in the presence of suitable hydrophobic molecules, leading to the formation of a single helical complex also known as inclusion complex. The formed helix has a hydrophobic interior that interacts with non-polar molecules and a hydrophilic exterior.[1,2]

Functional compounds that help to prevent and treat different diseases are known as nutraceuticals. The nonelectrolyte or weak electrolyte nature of these compounds contributes to their low water solubility[3], thus resulting in a reduced bioavailability and difficulties in the exploitation of their properties. Naringenin is a nutraceutical compound that is naturally present in citrus fruits (grapefruit, orange) and tomato[4] and exhibits antioxidant[5], lipid peroxidation protection[6] and antiatherogenic activity[7]. Yet its use is limited due to its poor water solubility and bioavailability. Therefore, there are attempts to improve naringenin solubility, such as complexation with β-cyclodextrin and its derivatives[8-10], phospholipids[11], and β-lactoglobulin.[12] These studies report an improvement in naringenin solubility, but the reported cylodextrin

toxicity[9,10], the tendecncy of phospholipids to aggregate[11], and the limited binding sizes in β-lactoglobulin represent a major concern.[12]

Starch inclusion complexes have been shown to exert protective action over guest molecules and increase their solubility[13-15]. Complexes of starch with fatty acid esters of bioactive compounds[16], ferulic acid[17], and genistein[18] displayed an increase in the inclusion rate, although the complexation yield remained low, which was attributed to the tendency of native starch to easily re-associate. Recently, Zhu & Wang[19], Arijaje et al.[20], and Arijaje & Wang[21] demonstrated that chemical and enzymatic modifications were effective in improving starch complexation with guest molecules such as α-naphthol, stearic acid, and oleic acid.

The goal of this study was improve the formation of inclusion complex between modified starches and naringenin. It was hypothesized that both starch structure and chemical modification impacted starch complexation capability with naringenin. The specific objectives of this study were 1) to investigate the effects of acetylation and hydroxypropylation in combination with a β-amylase treatment on starch of different botanical sources to form complexes with naringenin, and 2) to characterize the physicochemical properties of the soluble and insoluble starch-naringenin complexes. The development and characterization of starch and naringenin complexes may lead to a better understanding of the interaction between starch and naringenin and also to the formulation of naringenin for healthy food or non-food products.

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CHAPTER 2

LITERATURE REVIEW

2.1 Starch

Starch is the second most abundant carbohydrate, after cellulose, in plants. It is widely used as a raw material for various industries and plays an important role in human diet because of its prevalent presence such as in seeds, roots, tubers, and fruits.[1] The versatility of starch is attributed to its chemical structure, composition, and granular arrangement, which vary greatly from source to source.[2] The main differences among major commercial starch sources are summarized in Table 2.1.

Table 2.1 Properties and Characteristics of Starch from Different Sources[3,4]

	maize	wheat	rice	tapioca	potato
source	cereal	cereal	cereal	coot	tuber
shape	round polygonal	round lenticular	angular polygonal	oval truncated	round oval
diameter (μm)	2-30	1-40	3-8	4-45	5-100
crystallinity type	A	A	A	A or C	B
amylose (%)	23-32	23-29	17-30	17-33	18-29
lipids (%)	0.6-0.8	0.3-0.8	0.3-0.4	0.03-0.1	0.02-0.2
proteins (%)	0.3-0.4	0.3	0.5	0.2	0.1-0.4
phosphate (nmol/mg)	0.11	0.20	0.12	1.11	23.2

Starch is composed of two homopolymers, amylose and amylopectin, made of anhydroglucose units linked by α-D-(1→4) glucosidic linkages. Amylose is essentially linear, while amylopectin has 4-5% branching through α-D-(1→6) glucosidic linkages. The amylose-amylopectin ratio is characteristic of the source: ~20-35% amylose in most starches, close to 0% in waxy starches, and >40% in high amylose starches. Although the minor components such as lipids, proteins, and minerals only constitute 1-2% in starch, they exert great impacts on starch

properties. For example, the high phosphate monoesters in potato starch (Table 2.1) yield pastes with high clarity and viscosity upon gelatinization.[2,5]

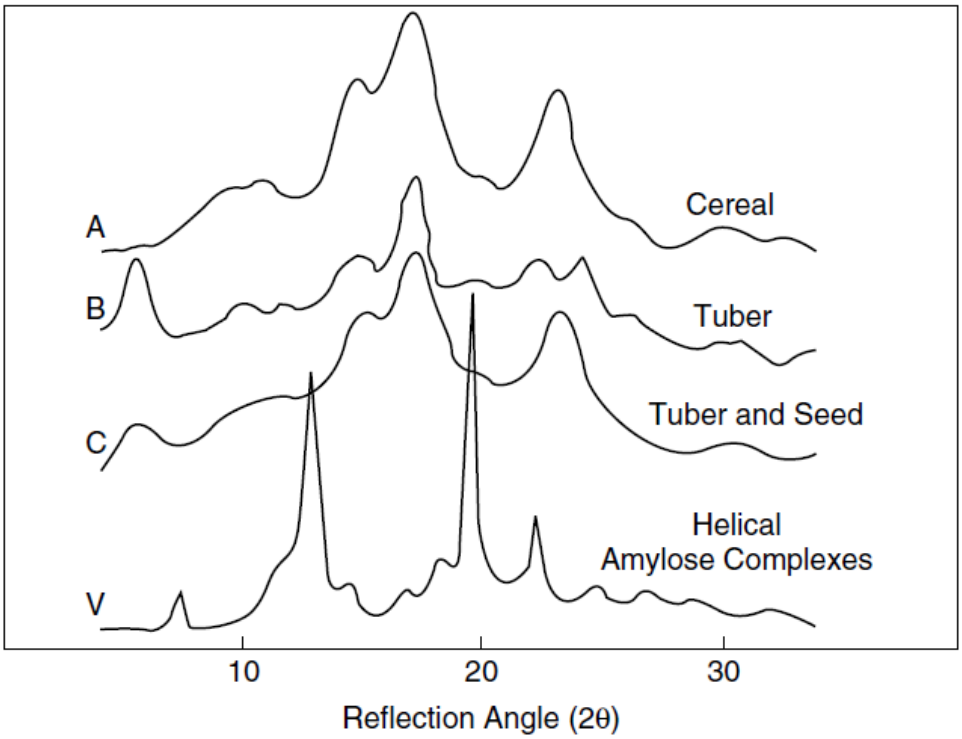

Figure 2.1. X-ray diffraction patterns of A, B, C, and V type starches.[6]

The relationship between crystalline and non-crystalline structures greatly influences starch properties. Starch has a distinctive X-ray diffraction pattern (Figure 2.1) because of the polymeric forms of starch crystalline structures. Cereal starches exhibit the A-type pattern, tuber starches display the B pattern, and some other tubers, seeds and roots yield the C-type pattern. The differences among them obey to the geometry of the unit cells, density, bounded water, and chain length (CL) distribution. The A-type pattern having less water (8 water molecules per unit cell) is highly dense and generally presents strong peaks at reflection angles (2θ) 15.3°, 17.1°, 18.2°, 23.5°. Starches with the B-type pattern have a hexagonal unit cell and more water per unit cell (36 water molecules) with strong peaks at 5.59°, 14.4°, 17.2°, 22.4°, 24.0°. Shorter average chain lengths (CL ~26) generally yield the A-type pattern, whilst the B-type pattern is associated

with longer average chain length (CL ~36). C-type pattern is considered a mixture of A and B patterns, but it also has been considered as a different structure rather than a mixture. The C-type pattern shows strong peaks at 3.73°, 15.3°, 17.3°, 23.5° and is associated with intermediate average chain length (CL ~28).[6-8] The V-type pattern is induced by complexation of amylose with non-polar molecules and some organic molecules such as butanol, and displays strong peaks at 7.8°, 13.5°, 20.9°.[6,9]

2.1.1 Amylopectin

Amylopectin is a large molecule with 4-5% branching and molecular weight ranging between 10^6 and 10^9 g/mol and degree of polymerization (DP) by number (DP_n) from 9,600 to 15,900 glucose units.[10-12] The molecular structure of amylopectin has been widely accepted as a cluster model (Figure 2.2) first introduced by French[13] and refined by Hizukuri.[14,15]

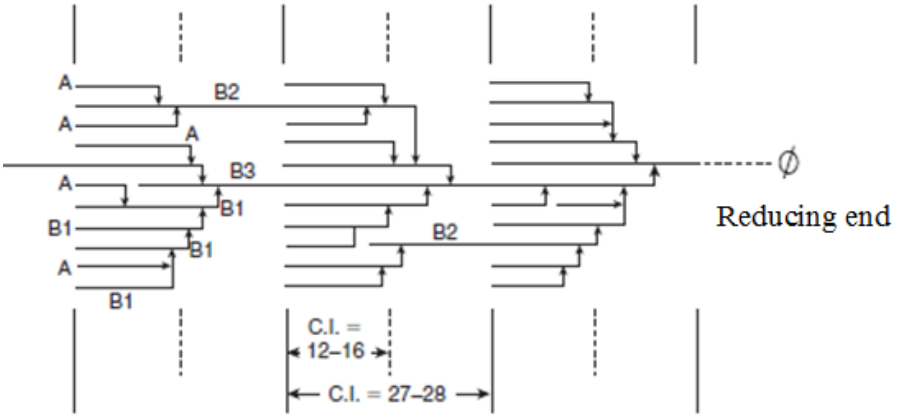

Figure 2.2. Amylopectin cluster model.[15]

Amylopectin chains are classified into three main categories according to their CL and position. A chains are unsubstituted by other chains; B chains are attached to A and other B chains; C chain, only one C chain in each amylopectin, carries the only reducing group of the molecule. Hanashiro et al.[16] further refined the polymodal distribution of amylopectin chains as A chains with DP 6-12, B1 chains with DP 13-24, B2 chains with DP-25-36, and B3 with DP

>37. B1 are chains connecting just one cluster, while B2 and B3 chains are connected to two or three clusters, respectively.

2.1.2 Amylose

Amylose is an essentially linear polymer with molecular weight approximately 10^5-10^6 g/mol, and DP_n 324-4,920 glucose units, which vary with botanical source. For example, high amylose maize starch exhibits considerable smaller molecular weight with an average DP 690-740 and CL 215-255[17], while potato starch exhibits a DP 840–21,800 and CL 670.[18] The slight branching nature of amylose has been demonstrated by its incomplete hydrolysis when subjected to β-amylase hydrolysis.[17,19]

Amylose tends to re-associate to become a more ordered state through hydrogen bonds.[20] The linear nature of amylose chains promotes their rapid aggregation and subsequent double helices formation, which is favored with DP around 100.[21] The double helices adopt a left-handed form with either A or B diffraction pattern.[22-24] In aqueous solution amylose adopts a random coil structure, which is unstable and tends to form helical complexes in the presence of molecules such as iodine, monoacyl lipids, alcohols, and flavor compounds. The formed helix has a hydrophobic inner cavity due to the aliment of glycoside linkages with methylene groups, whilst the hydroxyl groups are oriented toward the exterior to confer a hydrophilic exterior, left-handed orientation, and depending on the size of the ligand 6, 7, or 8 glucose units per turn. The hydrophobic nature of the helix inner cavity allows the inclusion of hydrophobic ligands.[18,25-28]

2.2 Naringenin

Naringenin (4',5,7-trihydroxyflavanone, Mw 272.26 g/mol) (Figure 3) is a major flavanone present in citrus fruits.[29] Shirasaka et al.[30] reported a naringenin concentration of 0.0730 μM in grapefruit juice and 0.0101 μM in orange juice. Naringenin is soluble in solvents

such as ethanol, and because of its weak acid character (p*K*a 6.7) naringenin solubility is greater in basic media.[31,32]

Naringenin has been shown to exhibit anti-inflammatory, anticarcinogenic, antitumor, and antioxidant activity.[33,34] However, because of the hydrophobic ring structure, as many other flavonoids, naringenin exhibits low water solubility, reduced bioavailability, and unfavorable pharmacokinetic, which restrict its potential use as biopharmaceutical.[35] Shulman et al.[36] reported naringenin solubility as 36.1 μM, whilst Löf and Nilsson[37] described a rapid crystallization of naringenin when transferred from an organic solvent to an aqueous system.

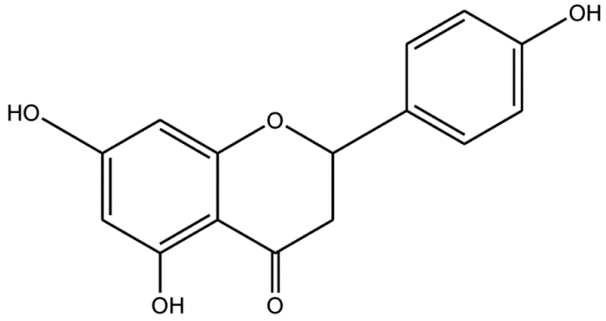

Figure 2.3. Naringenin chemical structure.[38]

2.3 Cyclodextrin Inclusion Complexes

Cyclodextrins are oligosaccharides derived from starch with a cyclic structure of 6 (α), 7 (β) or 8 (γ) glucose units, respectively, linked by α-D-(1→4) linkages. Cyclodextrins are characterized of having a truncated cone shape (Figure. 2.4) with a hydrophilic surface from secondary and primary hydroxyl groups and a hydrophobic inner cavity through hydrogen atoms and glycosidic oxygen bridges, allowing the formation of inclusion complexes with non-polar molecules.[39,40] The ability of cyclodextrins to form inclusion complexes through host-guest interactions is related to various driving forces, including hydrogen bonding, Van der Walls, electrostatic interactions, and substitution of water molecules by the guest. The most common

host to guest ratio is 1:1, although more complex associations (2:1, 1:2, 2:2) are possible depending on the guest molecular structure.[41,42]

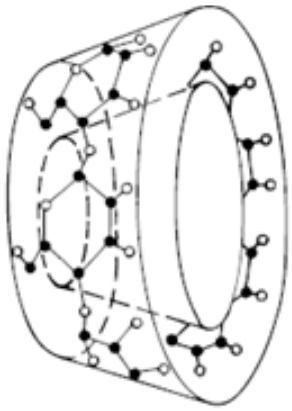

Figure 2.4. Cyclodextrin truncated cone shape.[49]

Cyclodextrins have been shown to increase the solubility and bioavailability of phenolic compounds such as cathechin and quercetin[43], and ferulic acid.[44] Nevertheless, cyclodextrins present limitations due to their low solubility in water and organic solvents. Derivatives, particularly from β-cyclodextrin, with enhanced physicochemical properties have been prepared, including methyl, hydroxypropyl, acetyl, and sulfobutyl- β-cyclodextrin, through substitution of hydroxyl groups.[45,46] Yatsu et al.[47] reported improved solubility of genistein by methyl-β-cyclodextrin when compared with β-cyclodextrin. The complex of naringenin with hydroxypropyl-β-cyclodextrin also has been shown to exhibit 400x increase in naringenin solubility.[36] Yang et al.[48] also reported that the complex of naringenin with heptakis-(2,6-di-O-methyl) (DMβCD) and heptakis (2,3,6-tri-O-methyl) (TMβCD) β-cyclodextrins increased not only naringenin thermal stability, but also solubility from 4.38 μg/mL to a maximum of 1.60 mg/mL. Nevertheless the high cost associated with cyclodextrin modification and the restrictions of the cavity size make the application of cyclodextrin limited.

2.4 Amylose Inclusion Complexes

The ability of starch, especially the amylose fraction, to form inclusion complexes was first described by Colin and Claubry[50] as a function of amylose reaction with iodine. Katz and Itallie[51] found an X-ray diffraction pattern in bread baking and also in the reaction of starch with alcohols, i.e. V-pattern, which is different from the A and B forms of the native starch. Amylose complex can be described as the combination of a ligand within the ligand-induced amylose helix with a central hydrophobic cavity and six to eight glucoses per pitch, which is the distance between identical points in two sequential turns (Figure 2.5).[52,53]

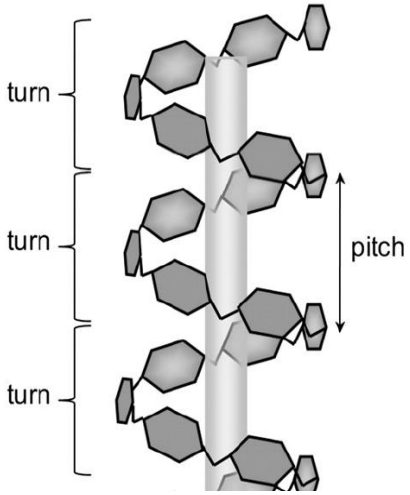

Figure 2.5. Amylose helix organization.[54]

The complexation between amylose and iodine has been used to determine the apparent amylose content based on the intensity of the blue color formed. The color intensity depends on amylose chain length; chain lengths above 80 are required to produce the intense blue color, and the color shifts from purple to brown as the chain length decreases.[55] Using the above mentioned principle, amylose has been used to form complexes with fatty acids[56], flavors[57], and aroma compounds[58], demonstrating the influence of inclusion complexes in the resistance of starch to

amylase hydrolysis, pasting properties, and retention or release of volatile compound during processing.

2.5 Factors Influencing Amylose-Inclusion Complex Formation

2.5.1 Ligand Concentration and Solubility

The ability of a guest molecule to form complexes with starch is affected by its size, shape, and hydrophobicity. Molecules with long hydrocarbon chains are believed to be included inside the helix, while short linear molecules such as fatty acids (C-8) or lactones (C-7) form unstable complexes.[59-61] At low ligand ratio it has been suggested that the amylose-amylose interactions prevail over the interactions amylose-ligand, forming double helices of retrograded amylose.[62,63]

The ligand solubility is affected by the solvent. Polar solvents such as methanol and ethanol are commonly employed to solubilize the ligand prior to the complexation reaction. The host-guest interaction is enhanced when the ligand is dissolved in the solvent before adding to the starch solution.[64] The solvent should help to increase the ligand intrinsic solubility but not compete for the amylose cavity.[65] Likewise, changes in ligand pH also alter ligand solubility. For example, changes in the pH of phenolic compounds cause ionization of the ligand, which greatly affects its intrinsic solubility and hydrophobicity. Jovanovic et al.[32] suggested that changes from uncharged to charged forms of some flavonoids induced different behavior: the ionized forms tended to be more soluble, while in neutral state the hydrophobic character prevailed. Tommasini et al.[66] used cyclodextrin to complex naringenin, and reported that when the reaction occurred at pH 4 the hydrophobic character of naringenin prevailed and a more stable complex was formed due to the affinity of naringenin for the hydrophobic cavity. In contrast, at pH 8 the solubility in water increased but the stability of the complex was lower. A similar behavior of genistein-

amylose complex was described by Cohen et al.[67], which suggested that at a pH as high as 8.0 the dissolution of the complex was feasible due to an increase in the interaction between water and genistein.

2.5.2 Starch Degree of Polymerization (DP)

The complex formation has been attributed particularly to amylose chains, but amylopectin long (DP ≥ 20) external branches also contribute to the complex formation.[68] The effect of amylose chain length has been extensively studied for amylose-fatty acid complexes, concluding that a DP around 20–400 is necessary to include fatty acids in the helix.[60,69,70] Cohen et al.[67] suggested that a minimum DP of 13 was required to form inclusion complex of amylose with genistein, an isoflavone with similar structure to naringenin.

2.5.3 Incubation Temperature and Time

Temperature plays an important role in amylose-inclusion complexes formation. Ahmadi-Abhari1 et al.[56] investigated the effects of incubation temperature and time on amylose-lysophosphatidylcholine (LPC) complex formation, and demonstrated that heating the solution for 1 h increased the amount of complexed LPC from 18.7% at 20°C, to 48.8% at 50-60°C. The study concluded that at temperatures below starch gelatinization temperature complex formation was feasible but required longer times. Nevertheless, the extent of complexation increased when the reaction temperature increased from 20 to 50°C, which was attributed to an increase in amylose mobility and partial loss of the crystalline structure. On the other hand, Chang et al.[71] studied the complex of conjugated linoleic acid (CLA) with linear dextrins obtained from debranched waxy corn starch, and concluded that increasing the temperature from 30 to 60°C decreased the amount of complexed CLA from 1.67% to 1.21%. An increase in temperature may lead to a better solubility of both starch and guest molecule, enhancing the complex formation;

however excessive heat can also destabilize the formed complex. The optimum temperature for complex formation depends on guest structure and thermal stability.

2.6 Starch Modification

Starch is an abundant natural resource with advantages of being biodegradable, environmentally friendly, and cost effective.[72] Nevertheless, the industrial application of native starch is limited because of many undesired properties, such as its tendency to retrograde, instability at low pHs and high temperatures, and low water solubility. Approaches to overcome these problems and meet the requirements for various applications include physical, chemical, enzymatic, and genetic modifications. Chemical modification has been shown to reduce starch tendency to retrogradation and to increase solubility, while enzymatically-treated starch may offer a more linear and favorable structure capable of forming inclusion complexes to a greater extent.

2.6.1 Chemical Modification

Substitution.

The introduction of functional groups is employed to modify starch properties such as gelatinization, pasting, and retrogradation through replacement of hydroxyl groups with functional groups. The degree of substitution (DS), which represents the number of hydroxyl groups substituted per anhydroglucose unit (AGU), for commercial starches is usually less than 0.2.[73] The maximum DS that can be achieved is 3 because only C2, C3, C6 are available for reaction with functional groups.

Acetylation. Acetylation is one of the most common substitutions and is commonly achieved through esterification of native starch with acetic anhydride or vinyl acetate in the presence of alkaline catalysts such as NaOH, KOH, or Na_2CO_3 (Figure 2.6).[74-76] The introduction

of acetyl groups yields starches with increased swelling power and solubility, and decreased gelatinization temperature due to the reduction in hydrogen bond strength.[77,78] In general, the reaction is conducted in aqueous media in the presence of a salt (NaCl or Na_2SO_4) to prevent starch gelatinization, and under these conditions acetylated starch is commercially available at a low DS (0.05-0.2). FDA regulations only allow addition of acetic anhydride or vinyl acetate to achieve up to 2.5% acetyl groups; generally available products have DS less than 0.05.

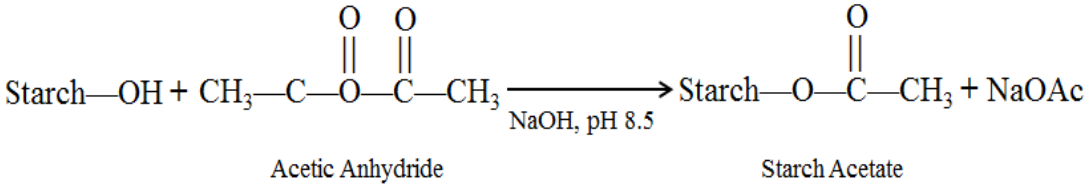

Figure 2.6. Acetylation of starch by acetic anhydride.

Hydroxypropylation. Hydroxypropylated starches are generally obtained by etherification of native starch with propylene oxide in the presence of an alkaline catalyst (Figure 2.7). The introduction of hydroxypropyl groups disrupts inter and intra-molecular hydrogen bonds, which weakens the granule structure and confers more mobility to the amorphous regions.[79,80] These starches are widely used in the food industry for their improved properties such as decreased pasting temperature, increased paste clarity, and improved freeze-thaw stability.[76] During hydroxypropylation, the substitution of hydroxy groups occurs mainly in the amorphous lamellae.[81] Reaction conditions and starch source affect the distribution of the substituents.[82]

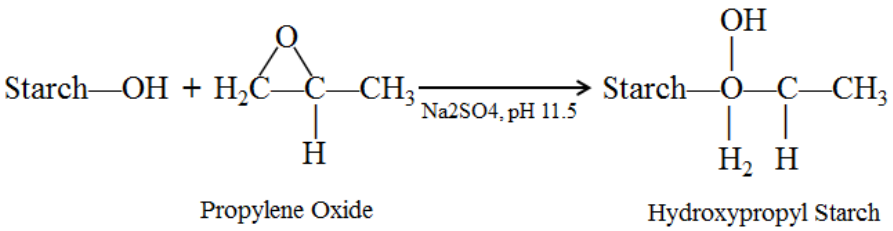

Figure 2.7. Hydroxypropylation of starch by propylene oxide.

2.6.2 Enzymatic Modification

Common enzymes employed in starch processing include α-amylase, β-amylase, glucoamylase, pullulanase, and isoamylase. During the hydrolysis process enzymes attack α-(1→4) and/or α-(1→6) linkages, depolymerizing starch into glucose, maltose, and/or oligosaccharides.[83]

Alpha-Amylase. Alpha-Amylase belongs to the endo-amylases family, and exerts action on α-(1→4) glucosidic linkages in the inner part of amylose and amylopectin chain. The action of this enzyme is not random and the final products, including branched oligosaccharides, are dependent on the source of the enzyme (microbial, plant or animal). Three different hydrolysis mechanisms have been proposed: single chain, multichain, and multiple attacks. The single chain mechanism, the enzyme catalyzes the reaction in a "zipper" towards until the end of the chain; in the multichain action the enzyme hydrolyzes only one bond per active encounter; for the multiple attacks mechanism the enzyme may hydrolyze multiple bonds per encounter.[84]

Beta-Amylase. Beta-amylase is an exo-amylase and hydrolyzes starch α-(1→4) glucosidic linkages from the non-reducing ends inward, producing maltose and β-limit dextrin. The hydrolysis of amylopectin by β-amylase only reaches 50 to 60 % because of its inability to bypass α-(1→6) linkages, and therefore chains of reduced molecular weight containing branching points are produced.[85]

Pullulanase-Isoamylase. Both enzymes hydrolyze α-(1→6) glucosidic linkages from amylopectin inner part, producing linear oligosaccharides. The difference between them: the chains that can be hydrolyzed. Isoamylase cleaves amylopectin chains with a DP of at least three, while pullulanase exert action amylopectin chains with a DP of at least 2 and β-limit dextrin.[86]

2.7 Modified Starches and Inclusion Complexes

Starch is capable of forming inclusion complexes with hydrophobic molecules, however native starch displays limited capability because of its tendency to retrograde and the highly branched structure of amylopectin. Efforts to enhance starch complexing ability have been carried out through chemical and enzymatic modifications. Wulff and Kubik[87,88] and Kubik and Wulff[89] demonstrated that hydroxypropylation (DS 0.075) of amylose allowed the formation of soluble complexes with 4-tert-butylphenol. They concluded that hydroxypropylation of amylose at DS 0.06-0.075 was sufficient to from soluble inclusion complexes with organic molecules, although more hydrophobic compounds required higher degrees of substitution (DS ≥0.13). Later, Kubik et al.[90] suggested that acetylated amylose complexing capability with iodine was slightly decreased, compared to hydroxypropylated amylose, when the DS increased. Wulff et al.[91] also compared acetylated and hydroxyproylated amylose at similar DS levels (0.04-0.61), and in both cases the complexing capability with fenchone slightly decreased when the DS increased, which was attributed to the repulsive effect of acetyl and hydroxypropyl groups impeding the helical configuration.

Enzymatic modification of starch has been used to increase the linear starch content, thus increasing complexing capability. Yotsawimonwat et al.[92] investigated the effect of pH and debranching on complex formation of waxy rice and fatty acids (FA) with different chain lengths and saturations, and suggested that FA (10:0-18:0) complexed to a greater extent with long linear starch chains as measured by a decrease in iodine absorbance of the complexes. Zhu and Wang[93] investigated the effect of chemical (hydroxypropylation and acetylation) and enzymatic modifications on the complex formation of high-amylose maize with α-naphthol, and concluded that both modifications considerably decreased the complex yield from 62.6% with Hylon VII to

27.5% in low acetylated Hylon VII. They suggested that this behavior may be attributed to the cyclic structure of α-naphtol and to the disturbance of helix formation by acetyl and hydroxypropyl groups. Van Hung et al.[94] reported an increase in ferulic acid solubility and antioxidant capacity when complexed with debranched starch. They also found that an increase in the ratio of ferulic acid to starch resulted in increased ferulic acid in complex from 6.8 to 32.5 mg/g of starch, concluding that there was an optimum ratio of guest molecule to starch for enhanced complexation. Recently Klaochanpong et al.[95] investigated complexes of debranched waxy corn, waxy rice, and waxy potato starches with iodine and fatty acids, and demonstrated that starch form different sources differed in their complexing capability, and the ability of starch to form complex with iodine increased after debranching. From all the starches studied, debranched waxy potato showed the highest complexing capacity for both fatty acid and iodine, presumably because of its longer chains.

Arijaje and Wang[96] studied the combined effect of chemical and enzymatic modifications on starch and oleic acid complexation, demonstrating enhanced formation of soluble and insoluble complexes. Acetylation significantly increased the recovery of soluble complexes, while the high-acetylated debranched starch showed the highest oleic acid in complex (38.0 mg/g). For insoluble complexes, low acetylated starch with or without the β-amylase treatment showed the highest complexed amount of oleic acid (37.6-42.9 mg/g). A similar trend was observed in a previous study for the complex of starch with stearic acid.[69]

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CHAPTER 3

ACETYLATION AND ENZYMATIC TREATMENT ON STARCH COMPLEXATION WITH NARINGENIN

3.1 ABSTRACT

Starch inclusion complexation has been shown to improve solubility of water insoluble molecules. This study prepared and characterized complexes of naringenin with potato starch and Hylon VII, which were acetylated at two levels and then debranched alone or combined with β-amylase hydrolysis. Both soluble and insoluble complexes were recovered and their physicochemical properties were characterized. The recovery of the soluble complexes and the naringenin content increased when starch was acetylated, and further increased when the β-amylase treatment was included. The insoluble complexes comprised a greater naringenin content than the soluble counterparts for both starches. The insoluble and soluble complexes of potato starch exhibited B+V and A+V X-ray patterns, respectively, whereas all complexes of Hylon VII displayed B+V type. FTIR spectra and thermal properties as measured by differential scanning calorimetry confirmed that both complexes were not physical mixtures. The results demonstrated that acetylation combined with enzymatic treatments improved starch complexation with naringenin.

KEYWORDS: acetylated starch, naringenin, β-amylase treatment, soluble complex, insoluble complex

3.2 INTRODUCTION

Bioactive compounds such as phenolics are naturally present in many food products and have been shown to provide potential health benefits and therapeutic effects in preventing chronic diseases such as cancer or diabetes.[1] Because of the number of aromatic rings and the position of the hydroxyl groups, many phenolic compounds exhibit low water solubility, instability to pH and temperature changes, and consequently poor bioavailability.[2] Naringenin is a phenolic compound naturally present in grapefruit and tomato skin and has been reported to exhibit anti-oxidant[3,4], anti-cancer[5], and antiatherogenic activities[6]. However, its therapeutic effects are limited due to its poor water solubility and bioavailability. Delivery systems have been developed to improve its solubility and bioavailability through different mechanisms. Shpigelman et al.[7] studied the binding between β-lactoglobulin and naringenin and suggested that the attachment of naringenin to the hydrophobic domains of β-lactoglobulin increased its solubility from 330 μM to 1000 μM. Nevertheless, the limited number of binding sites and the tendency to aggregate present a challenge of using β-lactoglobulin as a delivery system. Semalty et al.[8] improved the water solubility of naringenin from 161 μM to 291 μM by forming phospholipid complex of naringenin with phosphatidylcholine.

Cyclodextrins and amylose form inclusion complexes, which have a hydrophilic exterior and a hydrophobic interior where apolar guest molecules are situated.[9] The solubility of naringenin improved by at least 10-fold when complexed with β-cyclodextrin and its derivatives[10,11]; however, concerns remain in the poor water solubility and associated toxicity of cyclodextrins.[7] In contrast, starch chains are flexible, biodegradable, and biocompatible[11-13], although complexation is greatly affected by starch structure. Both common corn and waxy maize starches formed complexes with aroma compounds, indicating that amylopectin also

contributed to formation of complexes.[14] Gelders et al.[15] prepared amylose of degree of polymerization (DP) 20, 60, 400, and 950 in anhydroglycose units to form complexes with docosanoic acid and glyceryl monostearate, and concluded that the thermal stability of the complexes from amylose of DP 950 was lower than those of shorter DPs and that the minimal DP for complex formation was 35-40.

Starch inclusion complex is conventionally considered to be insoluble because of its crystalline nature. Wulff et al.[16] reported starch soluble complex when hydroxypropylated potato amylose with a degree of substitution (DS) 0.075 was used as a host to form complex with sodium dodecyl sulfate (SDS). Microcalorimetric and circular dichroitic investigations of this hydroxypropylated amylose demonstrated that the stability of the complexes was affected by amylose DP. Wulff et al.[17] reported that acetylated potato amylose (DP ~2600) with a DS 0.14 and lower resulted in insoluble complexes with fenchone, and that a minimum DS 0.16 was necessary to form soluble complexes. A noticeable decrease in the amount of complexed fenchone was observed for acetylated amylose with a DS 0.43 and greater. Recently Arijaje et al.[18] and Arijaje and Wang[19,20] demonstrated that acetylation and enzymatic modification of starch increased complex formation of starch with stearic, oleic, and linoleic acids. The formation of soluble complexes between debranched starch with stearic acid increased by 8.3% when starch was acetylated, and increased by 154% - 245% when the DP was reduced by β-amylase. A similar trend was observed with oleic and linoleic acid. In general, the recovery of insoluble complexes for these fatty acids decreased 23-52% as acetylation was combined with DP reduction. The amount of complexed fatty acids increased as the starch was acetylated and debranched[18-20]

Although β-cyclodextrin has been employed to improve naringenin solubility, little work has been done on complexation of starch with naringenin. Therefore, the objective of this study was to investigate the effect of acetylation and DP reduction on Hylon VII and potato starch for their complexing capability with naringenin. Both soluble and insoluble complexes were recovered and characterized for their physicochemical properties.

3.3 MATERIALS AND METHODS

Materials

High amylose corn starch (~70 % amylose, Hylon VII) and potato starch were gifts from Ingredion (Bridgewater, NJ). Isoamylase (specific activity 240 U/mg), pullulanase (specific activity 34 U/mg), and β-amylase from *Bacillus cereus* (specific activity 2,182 U/mg) were purchased from Megazyme Ltd. (Wicklow, Ireland). Naringenin was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals and reagents were of analytical grade. Because of its high inherent lipid content, Hylon VII was defatted by refluxing with 85% (v/v) methanol for 24 h, dried at 40°C for 24 h, and milled using a UDY Cyclone Mill (UDY Corporation, Ft. Collins, CO) fitted with a 0.5-mm sieve.

Acetylation of Starch

Potato starch and defatted Hylon VII were acetylated according to the procedure described by Wang and Wang[21]. Starch (100 g, db) was weighed into a 1-L reaction vessel and added with water to reach 35% solid (w/w). The mixture was hydrated for 30 min with stirring and added with 1 M NaOH to adjust the pH to 8.5. Acetic anhydride (4 or 8% based on starch db) was added dropwise while maintaining the pH at 8-8.5 using 1 M NaOH. The reaction was completed 60 min after the addition of acetic anhydride. The pH was adjusted to 5.5 with 1 M HCl, washed with a three-fold volume of deionized water, vacuum filtered through a Whatman

No. 1 filter paper, dried in a convection oven at 40°C for 24 h, and milled as described previously. The acetyl content was determined by the colorimetric method of McComb and McCready[22] and expressed as degree of substitution (DS) according to Wurzburg.[23]

Debranching of Starch

Native and acetylated starches were debranched by following the procedure of Arijaje et al.[18] with modifications. A mixture of 15 g (wb) and 200 mL deionized water (3.75% w/w) was heated in boiling water for 1 h to complete starch gelatinization. The solution was cooled down to 40°C, and adjusted to pH 5.0 with 0.5 M HCl. Isoamylase and pullulanase (1.33% each, starch db) were added, and the solution was incubated at 40°C for 48 h with stirring at 110 rpm. The debranched starch was precipiatated with four-fold volume of pure ethanol, centrifuged at 7000 ×g for 15 min, dried in a forced air oven at 40°C for 48 h, and milled using the UDY Cyclone Mill.

β-Amylase Treatment of Starch

After debranching and as indicated by the method of Arijaje et al.[18], the starch solution was adjusted to pH 6.5 with 0.5 M NaOH, β-amylase (0.5% v/w starch db) was added, and the solution was incubated at 40°C for 4 h with constant stirring. The reaction was terminated by boiling for 15 min, and the resultant starch was recovered by precipitating with 4-fold volume of pure ethanol and centrifuged as before. The resulting precipitate was dried at 40 °C for 48 h and milled using the UDY cyclone mill.

Preparation of Starch-Naringenin Complex

The method described by Arijaje et al.[18] was used with modifications to prepare starch-naringenin complexes. After debranching or debranching/β-amylase treatment, the starch solution (3.75% w/v) was adjusted to pH 7.0, and the temperature was gradually increased from

40 to 80°C. Naringenin (20 % starch db) that was previously dissolved in warm ethanol (6% w/v) was slowly added to the starch solution and allowed to react at 80°C for 30 min. The temperature was decreased to 45°C, and the reaction continued for 24 h with constant stirring. The slurry was centrifuged at 7,000 ×g for 15 min to recover insoluble complex in the precipitate and soluble complex in the supernatant. Both complexes were dried at 40°C for 48 h and then ground using the UDY Cyclone Mill. Uncomplexed naringenin was removed by vortexing both soluble and insoluble complexes (10% w/v) in 95% ethanol for 30 s and then rotating them using a labquake shaker (Barnstead/ thermolyne, Dubuque, IA) for 15 min. The resulting slurry was centrifuged at 20,000 ×g for 10 min, the supernatant was decanted, and a second wash was performed under the same conditions. The final precipitate was dried at 40°C for 24 h and then ground using mortar and pestle. The complex recovery was expressed as the complex weight recovered over the initial starch and naringenin weight.

Characterization of Inclusion Complexes

Starch Molecular-Size Distribution

High-performance size exclusion chromatography (HPSEC) was used to determine the molecular-size distribution of the debranched and debranched/β-amylase treated starches by following the method of Arijaje et al.[18] Ten mg of starch were solubilized in 5 mL of 90% dimethyl sulfoxide (DMSO), boiled for 1 h, stirred overnight at room temperature, and centrifuged at 9,300 ×g for 10 min before injection into a HPSEC system (Waters Corp., Milford, MA). The HPSEC system was equipped with an inline degasser, a model 515 HPLC pump with a 200-µL injector valve (model 7725i, Rheodyne, Cotati, CA), a model 2414 refractive index detector, a guard column (OHpak SB-G, 6.0 × 50 mm i.d × length), and two Shodex columns (OHpak SB-804 and KB-802, 8.0 × 300 mm i.d × length). The flow rate was

0.5 mL/min, and the mobile phase consisted of 0.1 M sodium nitrate with 0.02% sodium azide. The temperature of the columns was kept at 55°C and the detector at 40°C. The data was collected with an Empower Pro2 software (Waters Corp., Milford, MA), and the molecular-size distribution calculated by comparing against standards of molecular weight 180.16, 828.72, 1,153, 5,200, 148,000, 872,300, and 1,100,000 g/mol (Waters Corp., Milford, MA), and 1,100,000 g/mol (Sigma-Aldrich, St. Louis, MO).

Naringenin Content

Naringenin was released from the complexes by acid hydrolysis prior to analysis using a HPLC system (Beckman-Coulter, Fullerton, CA). Twenty-five mg of the complex was added with 2.5 mL of 1 M HCl and heated in boiling water for 1 h. The resulting solution was added with 12.5 mL of methanol and rotated overnight using the labquake shaker. A 1.5-mL aliquot of the solution was transferred to a 2-mL micro-centrifuge tube and centrifuged at 9,300 ×g for 10 min. A 0.5-mL aliquot of the supernatant was transferred to a glass vial with a screw cap. The reverse phase HPLC system was equipped with an autosampler (Model 508), a dual pump (model 126), and a photodiode array detector (Model 168). The analysis was performed using a Phenomenex (Torrance, CA) Kinetex XB-C18, 100A, 2.6 micron (100 × 4.6 mm) column, and a binary eluent of 0.1% trifluoroacetic acid in water as mobile phase A and acetonitrile as mobile phase B was run isocratically with 60% A and 40% B at a flow rate of 1 mL/min. Commercial naringenin (Sigma-Aldrich, St. Louis, MO) was used as a standard to identify and quantify the naringenin content in the complex. The data was collected using Beckman-Coulter System 32 Karat software (Version 8, 2006), and the results expressed as mg of naringenin per g of the complex.

Physicochemical Properties

Thermal Properties. The thermal properties of the complexes were analyzed using a DSC (Diamond, Perkin-Elmer, Shelton, CT). Approximately 3 mg of the complex was weighed into a stainless steel pan, and then 10 µL of distilled water were added. The pan was hermetically sealed and allowed to equilibrate at room temperature for 24 h before scanning from 25 to 200°C at 10°C/min. The onset temperature, peak temperature, end temperature, and enthalpy of any transition were calculated by Pyris data analysis software.

Wide Angle X-ray Diffraction Pattern. The X-ray diffraction pattern of the complexes was determined using a Philips PW 1830 MPD diffractometer (Almelo, the Netherlands). The X-ray generator was set at 45kV and the current tube at 40 mA. The scanning 2θ angle went from 5° to 35° with a step size of 0.02° at 1 s per step.

Attenuated Total Reflectance Fourier Transform Infrared (ATR FT-IR) Spectroscopy. The ATR FT-IR spectra of the complexes were recorded on a Nicolet 8700 spectrometer (Thermo Electron Scientific Inc., Waltham, MA) using a Golden Gate ATR accessory (Specac) equipped with a single-reflection diamond crystal. The temperature during measurements was kept at 25°C ± 0.1°C using an electronic temperature controller (Specac). For each spectrum, 64 scans were collected with a resolution of 4 cm^{-1}. The spectrometer's EverGlo source was on turbo mode during measurements. The spectrometer and ATR accessory were purged with dry nitrogen to diminish water-vapor contamination of the spectra. All samples were conditioned before their analysis for 7 days in a desiccator containing silica gel.

Statistical Analysis

Two replicates of the experiment were conducted, and each analysis was done in duplicate. The data was analyzed using JMP Pro13 Software (SAS Institute Inc., Cary, NC, USA), and the means compared using Tukey's honestly significant differences (HDS) test.

3.4 RESULTS AND DISCUSSION

3.4.1 Molecular Size Distribution

The molecular size distributions of native and acetylated potato starch and Hylon VII after debranching and after debranching/β-amylase treatment are presented in Figure 3.1. All debranched potato starches displayed four peaks (Figure 3.1A) at similar retention times (RTs) 20.6, 22.8, 27.9, and 29.2 min, corresponding to DP ~9,480, ~2,250, ~80 and ~32, respectively, but their proportions were different. Debranched Hylon VII showed three broader peaks (Figure 3.1B) at RTs 24.7, 27.9, and 28.9 min, corresponding to DP ~640, ~80, and ~39, respectively. Acetylation did not change the profiles of both starches; the β-amylase treatment resulted in starch chains of a narrower DP distribution with increased proportions of long DP chains for both acetylation levels of both starches (Figure 3.1 C and D). The molecular size distributions showed that potato amylose appeared at a shorter RT and consequently had a larger molecular size than Hylon VII amylose, agreeing with previous studies.[24-26] Nevertheless, the proportion of long DP chains was greater in Hylon VII. When considering all treatments, Hylon VII encompassed a large proportion of DP 40-650 chains, while potato starch contained a large proportion of DP 30-180 chains.

3.4.2 Degree of Substitution

The initial degrees of substitution (DS) of low (4%) and high (8%) acetylation were 0.049 and 0.095, respectively, for potato starch, and 0.037 and 0.094, respectively, for Hylon VII

prior to the enzyme treatment and complexation with naringenin. Both soluble and insoluble complexes were recovered and analyzed for DS. The soluble complex shared a similar DS with the insoluble complex within the same treatment for both starches (Table 3.1) . The β-amylase treatment resulted in starch with a significantly greater DS for both complexes because of the inability of β-amylase to bypass acetyl groups, agreeing with the findings of Arijaje et al.[18] and Arijaje and Wang.[19,20]

There was little difference in DS of acetylation between potato starch and Hylon VII for the same treatment, suggesting a similar distribution of acetyl groups in both starches possibly because of the rapid acetylation reaction[27], although their amylose contents were distinctly different. Under the conditions studied, soluble complexes were formed at a DS of 0.034 and 0.032 for potato starch and Hylon VII, respectively, which were lower than the DS reported for the formation of soluble complexes between acetylated potato starch and stearic (DS 0.063), oleic and linoleic acids (DS 0.048).[18-20] More planar molecules tend to be included in a greater proportion into the amylose helix, causing the complex to precipitate, hence a greater DS was required to maintain the soluble complex as demonstrated by Wulff and Kubik[16] and Wulff et al.[17] , which showed that hydroxypropylated amylose of DS 0.06-0.075 was needed to maintain soluble complexes with sodium dodecyl sulfate. The lower DS of the soluble complexes in the present study was attributed to the non-planar conformation of naringenin B-ring, which may favor the soluble complex formation.

3.4.3 Complex Recovery and Naringenin Content in Complex.

The total recovery for all treatments for both starches ranged 0.89-0.99 g/g (Table 3.1). There was no statistical difference between the recovery of potato starch and Hylon VII, although differences were observed among individual complex recovery. The recovery of the

soluble complexes increased as the DS of acetylation increased, displaying an increase by at least 100% for both acetylation levels. The decrease in recovery of the insoluble complex with increasing DS of acetylation was likely due to the disruption of the helical conformation by an increase in acetyl groups, agreeing with previous studies.[18-20] The incorporation of the β-amylase treatment did not significantly change the recovery of individual complex for both starches.

In general, the naringenin content in the insoluble complex (4.44–28.50 mg/g) was significantly greater than that in the soluble complex (2.35-10.86 mg/g) for the same treatment. Nevertheless, the naringenin content in the insoluble complexes increased with increasing DS of acetylation, differing from the work of Wulff et al.[17], in which the ability of acetylated amylose to complex iodine decreased with increasing DS from 0.16 to 0.43. The differences between these two studies were ascribed to the significantly greater DS in Wulff et al.[17] study, compared with the DS range of 0.031-0.080 for the insoluble complex in this study. Meanwhile, the naringenin contents in both complexes from Hylon VII were significantly greater than those from potato starch regardless of the treatment. Although the DS of acetylation was similar among starches for the same treatment, the greater complexing capability of Hylon VII is suggested to be related to its larger proportion of longer DP chains than potato starch, particularly chains of DP 180-650 (Figure 3.1).

The introduction of the β-amylase treatment generally increased the naringenin content in both soluble and insoluble complexes, except for the soluble complexes from low and high acetylated Hylon VII. The naringenin contents in the soluble complexes of potato starch (2.35-4.81 mg/g) were significantly lower than those of Hylon VII (6.00-10.86 mg/g), suggesting the importance of starch molecular size on the formation of complexes and the enhanced complexation from acetylation. In recent studies by Arijaje and Wang and Arijaje et al., the

maximum complexed stearic (123.1 mg/g)[18], oleic (42.9 mg/g)[19], and linoleic (54.4 mg/g)[20] acids were obtained from the insoluble complex of low acetylated/β-amylase treated potato starch. In contrast, the greatest naringenin content in the present study was from the insoluble complexes of high acetylated/β-amylase treated potato starch (11.75 mg/g) and Hylon VII (28.50 mg/g), indicating the important role of the guest molecule structure on the extent of complexation.

The available studies on the complexation of starch with phenolic compounds have not reported the effect of starch molecular size on the complexation yield.[28-33] The amylose DP involved in the complexation with fatty acids was suggested to be less than 400 but greater than 20.[24,34,35] Starch chains of DP >400 would not form a stable helix, but DP <20 was too short to induce the helical conformation. The present results indicate the importance of both acetylation and DP range on enhancing complexation, and there was a different combination of DS of acetylation and DP of starch chains for the formation of soluble complexes versus insoluble complexes. Starch chains of DP >400 is proposed to participate in complexation because Hylon VII comprised a greater proportion of DP >400 (Figure 3.1) and contained greater naringenin contents in all complexes compared with potato starch.

3.4.4 Physicochemical Properties of Starch-Naringenin Complexes

Wide Angle X-ray Diffraction Pattern

The X-ray diffraction patterns of potato starch and Hylon VII naringenin complexes are illustrated in Figures 3.2 and 3.3, respectively. The insoluble complexes from all treated potato starches displayed a B+V-type X-ray diffraction pattern (Figure 3.2), whereas the soluble complexes displayed an A+V or V-type pattern. The formation of A, B, or C type crystalline structure has been ascribed to be governed by the average chain length of amylopectin.[36,37] The A-type starches had a shorter average chain length (DP ≤19.7) than the B-type starches (DP

≥21.6). The present X-ray diffraction results suggest that longer DP chains tended to form insoluble complexes, whereas shorter DP chains favored the formation of soluble complexes, supporting the complexed naringenin results (Table 3.1) and the results by Arijaje et al.[18] and Arijaje and Wang[19,20]. The amorphous structure under the peaks generally increased as the DS of acetylation increased, confirming the interference of acetyl groups to the helical structure. Overall, potato starch complexes comprised a larger proportion of amorphous structure than Hylon VII complexes, inferring that shorter chains were less involved in the crystalline structure because a larger proportion of short chains was present in potato starch than in Hylon VII (Figure 3.1 A and B).

The V-type crystalline structure was observed in all soluble and insoluble complexes, with the soluble complex of high acetylated/β-amylase treated potato starch showing the most prominent V-type structure at diffraction angles ~13° and ~20°. The β-amylase treatment increased the intensity of the V-type crystalline structure of both soluble and insoluble complexes of potato starch, although the effect was not as significant as the complexes of stearic[18], oleic[19], and linoleic[20] acids. Unlike potato starch, the β-amylase treatment did not significantly affect the crystalline structure of Hylon VII complexes. Meanwhile, both soluble and insoluble complexes of Hylon VII showed a V+B-type structure (Figure 3.3), which was attributed to its larger proportion of long DP chains.

Kim and Huber[33] reported that the complex of β-carotene with common corn starch showed a V_h structure, which was not ascribed to the β-carotene complex but rather to the interaction of the alcohol used to solubilize β-carotene. They suggested that the configuration and steric hindrance of β-carotene did not allow the formation of a detectable crystalline structure. Quercetin and genistein are similar to naringenin in structure. The complex of

quercetin with common corn starch was highly amorphous[32], whereas the complex of genistein with Hylon VII exhibited a $V6_{III}$ crystalline structure, which was ascribed to guest molecule trapped between the helices.[29] Previous reports[38,39] on the complex of naringenin with β-cyclodextrins suggest that the phenyl ring in naringenin was more prone to entering the helix cavity, whereas the chromone moiety was oriented towards the exterior. It is hypothesized that the observed mixed crystalline structures in the present study represent a mixture of naringenin partly inside the helix and partly physically trapped between the helices inter-dispersed among varying proportions of short and long starch chains.

Attenuated total reflectance Fourier Transform Infrared (ATR FT-IR) Spectroscopy

Figures 3.4 and 3.5 shows the ATR FT-IR spectra of both soluble and insoluble complexes, the individual starches, and the physical mixture of naringenin with potato starch and Hylon VII, respectively. Before acetylation, the slight difference between the spectra of potato starch and Hylon VII lay in the location of the saccharide band at 992 cm^{-1} for potato starch and 998 cm^{-1} for Hylon VII, representing intra and inter molecular hydrogen bonds.[40] After acetylation, both starches showed strong absorption at 1720 cm^{-1} (C=O stretching of acetyl group), 1365 cm^{-1} (C–H in acetyl group) and 1244 cm^{-1} (C–O stretching of acetyl group).[41] These bands were characteristic of acetyl group attached to glucose units, and the bands increased with increasing acetylation level. Although no appreciable differences were noted between the spectra of the complexes for both starches, there were differences between the complexes and the physical mixtures, confirming that the results observed in the X-ray diffraction patterns were not physical mixtures, and that some association between starches and naringenin existed.

To better distinguish the differences in spectra, the differential spectrum of the complexes was analyzed by subtracting the corresponding starch band absorption from the complexes

spectra as well as the naringenin spectrum (data not shown). This analysis concluded that both starch and naringenin were involved in the complex formation. Bands between 3600-3000 cm^{-1}, which are related to free hydroxyl groups, were present in both differential spectrums. For acetylated starches the C=O stretching of the acetyl group (1720 cm^{-1}) remained unchanged. After the β-amylase treatment, both complexes showed decreased intensity on the aromatic band (1510 -1601 cm^{-1}), and the intensity of the –OH band of the phenyl ring (1200 cm^{-1}) was greater in the complexes of Hylon VII, but weaker in the potato starch complexes, which might be associated with the amount of complexed naringenin. The spectra results support X-ray diffraction results that both soluble and insoluble complexes existed in both amorphous and crystalline structures since the peak at ~1022 cm^{-1} was reported to be associated with the amorphous region of starch[42, 43] and the complexes displayed varied intensity at that band.

Thermal Properties by Differential Scanning Calorimetry

The thermal properties of all starch-naringenin complexes are summarized in Table 3.2. All insoluble complexes displayed melting endotherms, but only the soluble complexes of high acetylated starches displayed thermal transitions. Except for the insoluble complex of high acetylated debranched/β-amylase treated potato starch, the onset temperature of insoluble complexes decreased with increasing DS of acetylation, confirming previous studies that the introduction of acetyl groups interfered with re-association of starch chains and consequently less ordered crystalline structure.[18-20,44,45] The thermal transition of starch inclusion complexes, mostly from starch-lipid complexes, includes Type I complexes of a randomly oriented helical structure that dissociates between 95 and 105 °C and Type II complexes of aggregated Type I complexes melting around 115 °C.[46-48] Because starch and naringenin complexes comprised both

V-type and A- or B-type crystalline structures, their thermal transitions were broader than Type I complexes, suggesting a more heterogeneous nature of these complexes.

Similar to results by Arijaje et al.[18] and Arijaje and Wang[19,20], the enthalpy values for all complexes did not reflect the amount of complexed naringenin. The greatest naringenin content was obtained from the insoluble complexes of high acetylated β-amylase treated potato starch and Hylon VII with enthalpy values 6.5 J/g and 3.3 J/g, respectively. The absence of endotherms in the unacetylated and low acetylated soluble complexes is hypothesized to be associated with a crystalline structure comprising very small and isolated crystallites in the soluble complexes, which was easily destabilized and consequently not detected by DSC.

In conclusion, this study demonstrated that naringenin formed both soluble and insoluble complexes with debranched potato starch and Hylon VII. Acetylation increased the formation of the soluble complexes and the complexed naringenin, and the β-amylase treatment further enhanced the complexed naringenin. The molecular size distribution became narrower after the β-amylase treatment, which enhanced complexation with naringenin for both starches. Regardless the modification, starch structural characteristics determined the extent of complexation. Starch chains with DP >400 were suggested to be involved in complexing naringenin. Starch long DP chains encouraged the formation of insoluble complexes, where short DP chains were more involved in soluble complexes. Both soluble and insoluble complexes comprised a mixture of amorphous and crystalline structures as shown by their X-ray diffraction patterns. ATR FT-IR results confirmed the molecular interaction between starch and naringenin. It is important to consider both starch DP range and DS of acetylation for individual complex in order to maximize complexation yield with naringenin.

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Table 3.1. Degree of Substitution, Complex Recovery and Percentage of Naringenin Content Recovered from All Complexes[a]

debranched starch	acetylation	β-amylase treatment	type of complex	DS of acetylation	recovery (g/g)	naringenin in individual complex (mg/g)
potato	no	no	soluble	NA[b]	0.30±0.01D	2.45±0.01G
		no	insoluble	NA	0.64±0.00c	4.42±0.11e
		yes	soluble	NA	0.33±0.02D	2.67±0.33G
		yes	insoluble	NA	0.62±0.01c	3.91±0.25e
	low	no	soluble	0.034±0.055F	0.60±0.00C	2.35±0.76G
		no	insoluble	0.034±0.000f	0.31±0.00d	4.44±0.81e
		yes	soluble	0.049±0.022D	0.62±0.01C	4.00±0.34E-G
		yes	insoluble	0.045±0.022d	0.32±0.00d	6.69±0.77de
	high	no	soluble	0.071±0.044C	0.69±0.01B	3.90±0.33FG
		no	insoluble	0.067±0.000c	0.25±0.01e	7.53±1.10d
		yes	soluble	0.086±0.044A	0.71±0.03B	4.81±0.79D-F
		yes	insoluble	0.081±0.000a	0.25±0.04e	11.75±1.05c
Hylon VII	no	no	soluble	NA	0.12±0.00F	6.72±1.12CD
		no	insoluble	NA	0.87±0.00a	8.06±1.20d
		yes	soluble	NA	0.14±0.00F	8.82±0.07AB
		yes	insoluble	NA	0.83±0.00a	13.61±0.52c
	low	no	soluble	0.032±0.022F	0.24±0.00E	10.86±0.59A
		no	insoluble	0.031±0.022g	0.72±0.00b	11.92±0.67c
		yes	soluble	0.041±0.000E	0.24±0.00E	9.39±0.06A
		yes	insoluble	0.040±0.022e	0.65±0.00c	18.66±0.69b
	high	no	soluble	0.077±0.022B	0.77±0.00A	6.93±0.09BC
		no	insoluble	0.077±0.000b	0.19±0.00f	17.79±0.06b
		yes	soluble	0.089±0.022A	0.77±0.00A	6.00±0.23C-E
		yes	insoluble	0.080±0.000a	0.19±0.00f	28.50±0.00a

[a]Mean of two replicates with standard deviation. Mean values in the same column followed by different uppercase or lowercase letters are significantly different based on Tukey's HSD test. [b]NA: Not applicable.

Table 3.2. Melting Temperatures and Enthalpies[a] of Recovered Soluble and Insoluble Fractions of Native and Acetylated Potato Starch and Hylon VII Naringenin Complexes

debranched starch	acetylation	β-amylase treatment	type of complex	temperature (°C)			enthalpy (J/g)
				onset	peak	end	
potato	no	no	soluble	ND[b]	ND	ND	ND
		no	insoluble	101.0±1.1b	110.3±0.1b-d	118.2±0.63a-c	9.8±0.2a
		yes	soluble	ND	ND	ND	ND
		yes	insoluble	100.6±0.4b	109.9±0.1cd	118.3±0.36a-c	10.3±0.5a
	low	no	soluble	ND	ND	ND	ND
		no	insoluble	94.5±0.0c	105.9±0.1e	111.7±0.01e	3.2±1.0b
		yes	soluble	ND	ND	ND	ND
		yes	insoluble	94.4±0.5cd	107.0±0.0e	110.9±0.56e	3.6±0.7c
	high	no	soluble	90.5±0.7C	106.2±1.0A	117.0±0.41A	6.0±0.5AB
		no	insoluble	92.5±0.0e	109.8±0.5d	119.1±1.04ab	4.4±0.9bc
		yes	soluble	91.1±0.6C	99.0±0.8B	105.6±1.39C	4.1±0.9B
		yes	insoluble	108.6±0.1a	112.3±0.0a	118.4±0.47a-c	6.5±0.7c
Hylon VII	no	no	soluble	ND	ND	ND	ND
		no	insoluble	101.1±0.4b	111.9±0.0ab	119.2±0.35ab	3.1±0.2
		yes	soluble	ND	ND	ND	ND
		yes	insoluble	100.6±0.1b	111.8±0.0a-c	120.2±0.73a	5.2±0.1bc
	low	no	soluble	ND	ND	ND	ND
		no	insoluble	102.0±0.1b	111.1±0.7a-d	117.6±0.62bc	3.6±0.3c
		yes	soluble	ND	ND	ND	ND
		yes	insoluble	102.0±0.7b	111.0±0.4a-d	118.3±0.45a-c	4.2±0.0c
	high	no	soluble	99.5±0.6A	106.6±0.2A	113.7±1.82AB	7.1±0.4A
		no	insoluble	92.3±0.7e	109.7±0.6d	115.2±0.54d	3.9±0.1c
		yes	soluble	93.3±0.1B	104.6±0.0A	111.2±0.93B	6.7±0.8AB
		yes	insoluble	92.5±0.1de	110.2±0.8b-d	116.4±0.08cd	3.3±0.9c

[a] Mean of at least two replicates with standard deviation. Mean values in the same column followed by different uppercase or lowercase letters are significantly different based on Tukey's HSD test. [b]ND: Not detected

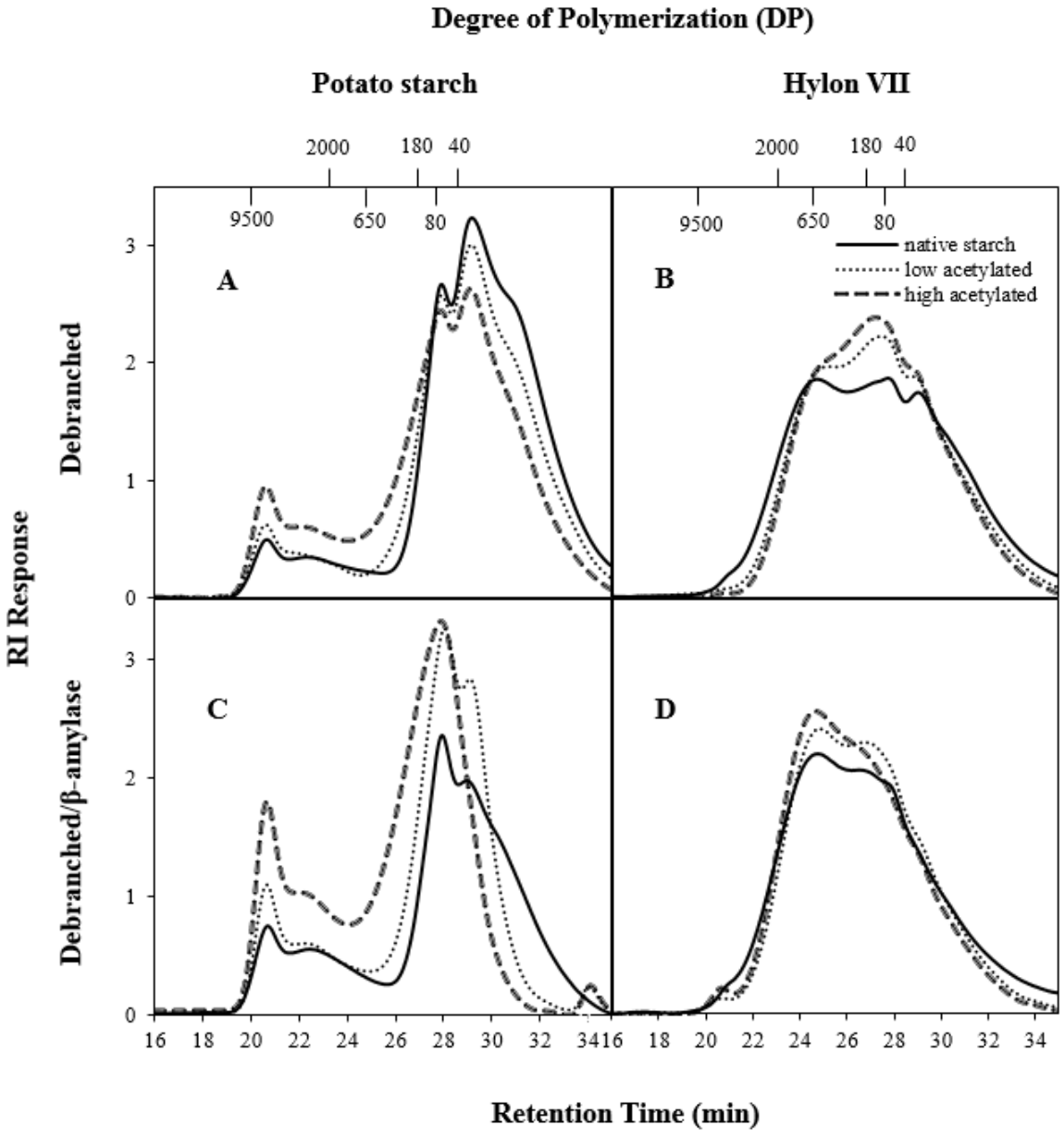

Figure 3.1. Normalized size-exclusion chromatograms of native and acetylated debranched and debranched/β-amylase treated potato and Hylon VII starches.

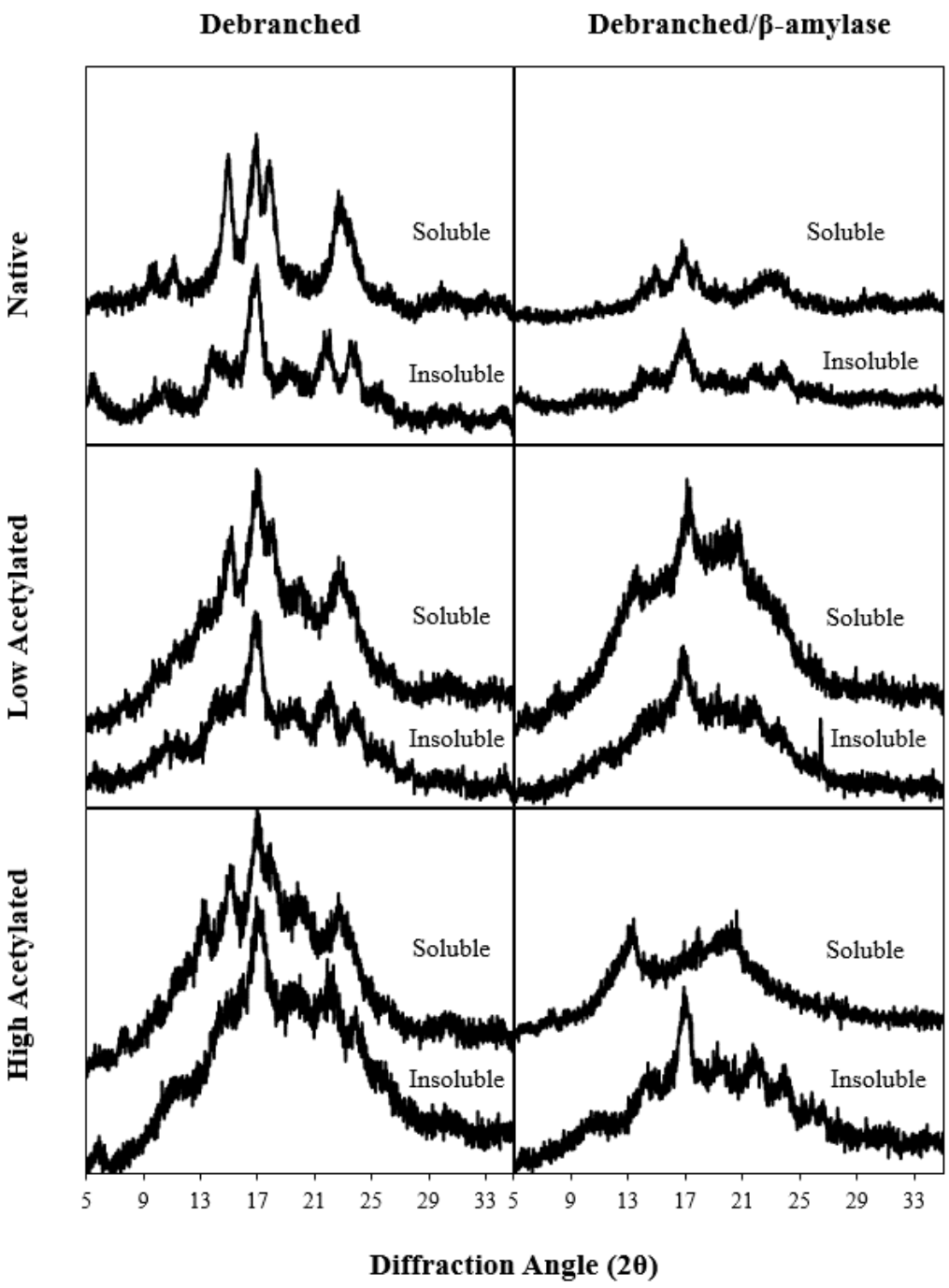

Figure 3.2. X-ray diffractograms of soluble and insoluble starch-naringenin complexes of native and acetylated debranched and debranched/β-amylase treated potato starch.

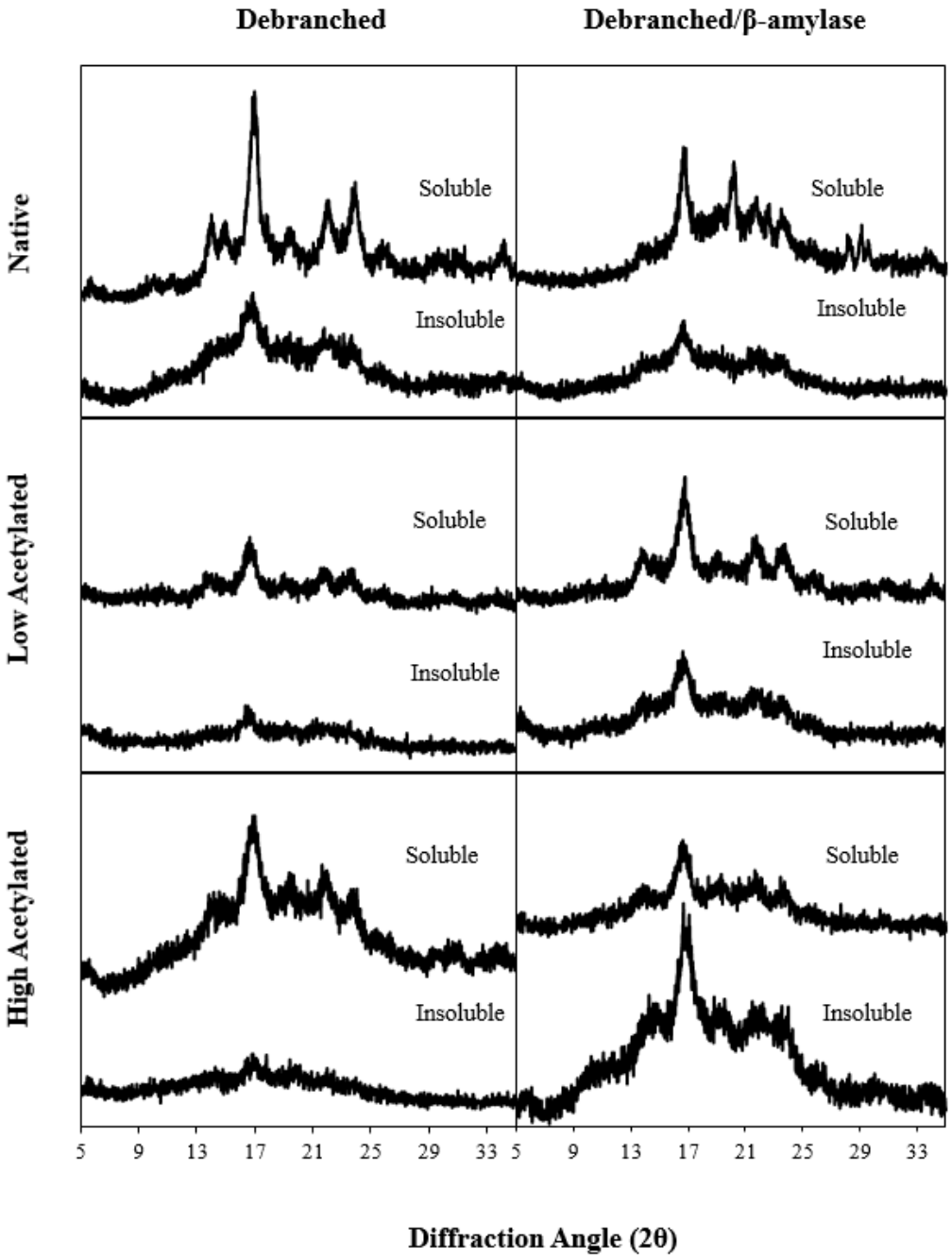

Figure 3.3. X-ray diffractograms of soluble and insoluble starch-naringenin complexes of native and acetylated debranched and debranched/β-amylase treated Hylon VII.

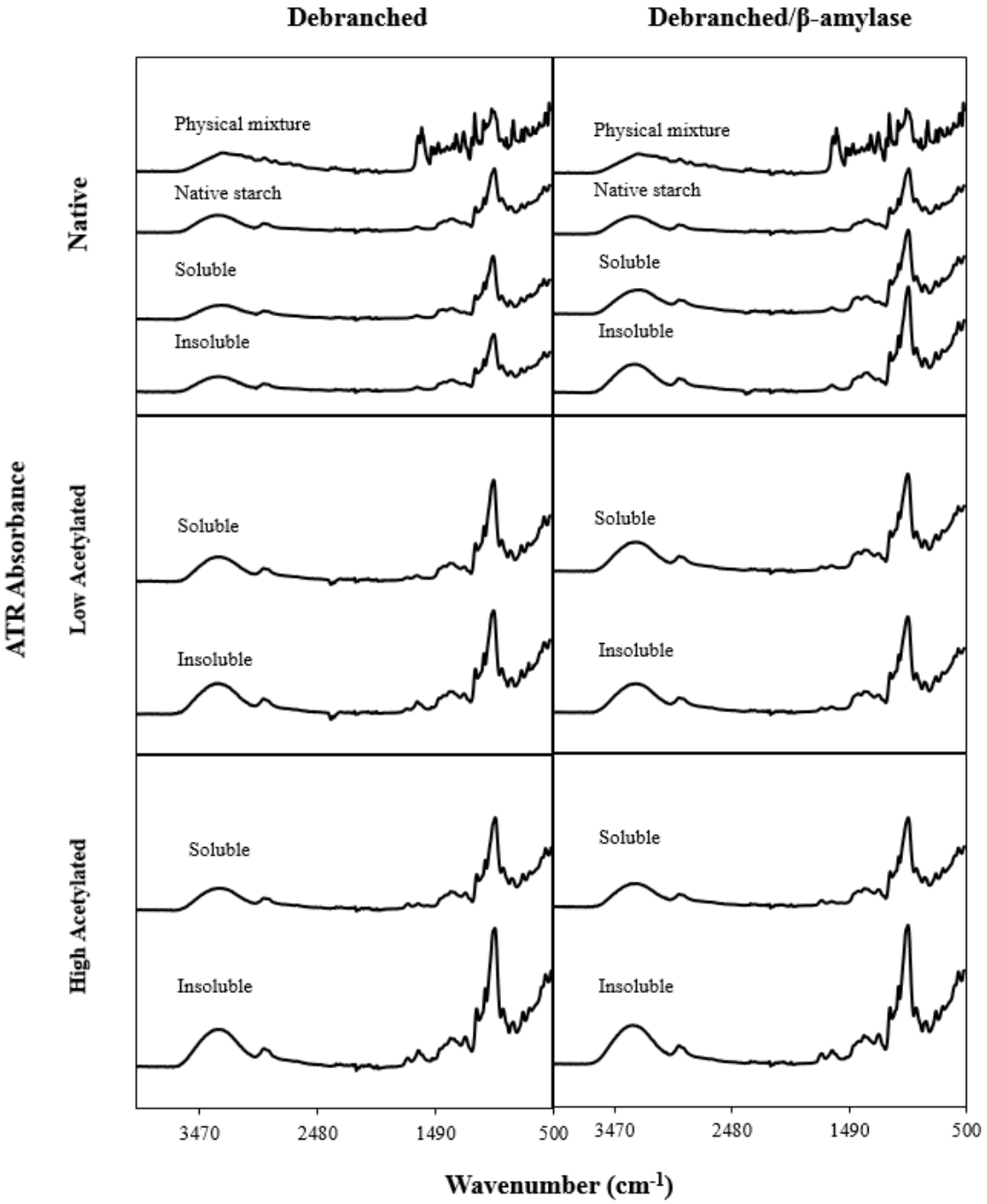

Figure 3.4. Attenuated Total Reflectance Fourier Transform Infrared (ATR FT-IR) spectra of soluble and insoluble starch-naringenin complexes of native and acetylated debranched and debranched/β-amylase treated potato starch. The spectra of native potato starch and physical mixture of native potato starch and naringenin are included for comparison.

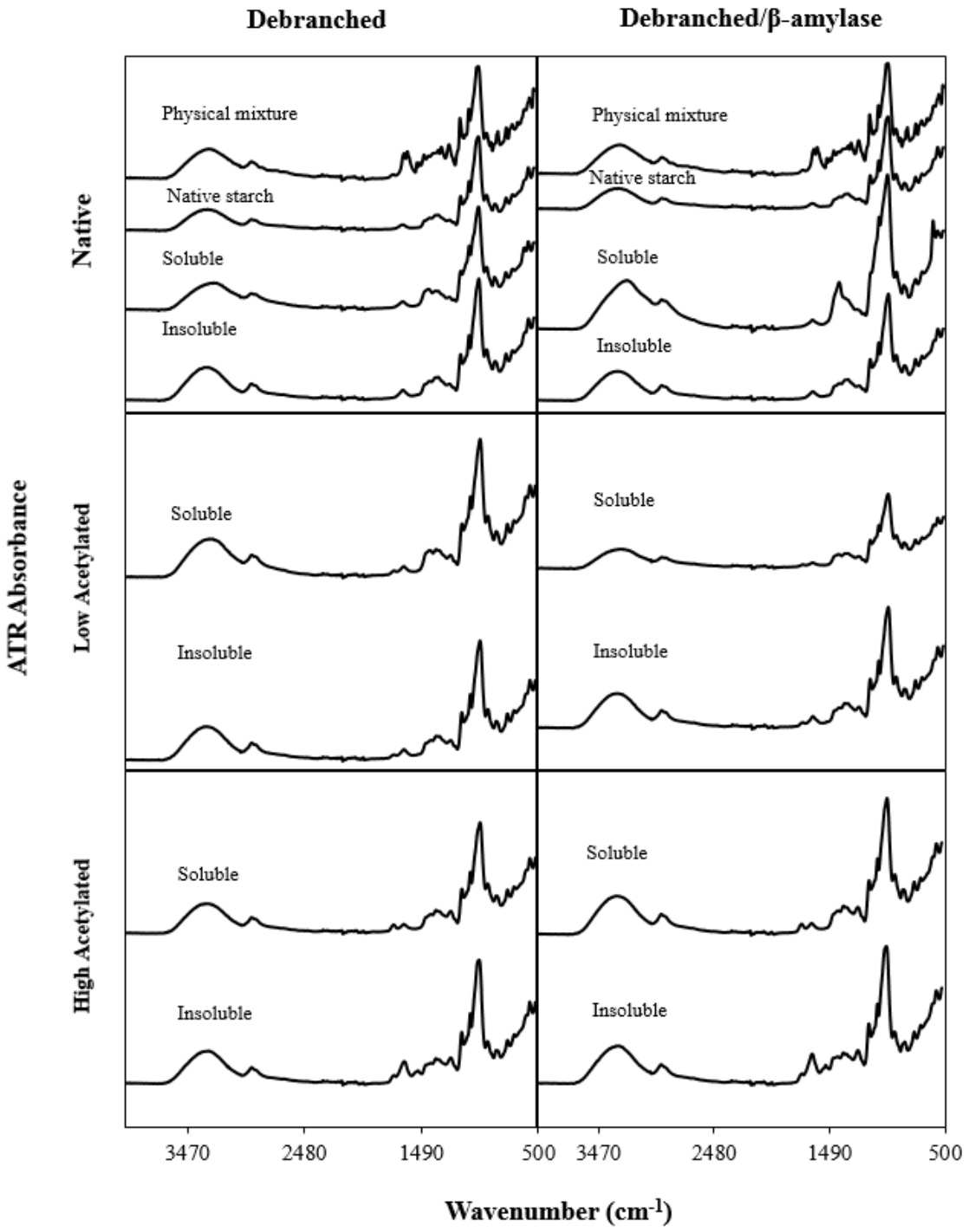

Figure 3.5. Attenuated Total Reflectance Fourier Transform Infrared (ATR FT-IR) spectra of soluble and insoluble starch-naringenin complexes of native and acetylated debranched and debranched/β-amylase treated Hylon VII. The spectra of native Hylon VII and physical mixture of Hylon VII and naringenin are included for comparison.

CHAPTER 4

HYDROXYPROPYLATION AND ENZYMATIC TREATMENT ON STARCH COMPLEXATION WITH NARINGENIN

4.1 ABSTRACT

The objective of this study was to investigate the effect of hydroxypropylation and enzymatic treatments on starch complexation with naringenin. Potato starch and Hylon VII were hydroxypropylated to two degrees of substitution and then debranched or debranched/β-amylase treated prior to complexing with naringenin. Both soluble and insoluble complexes were recovered and characterized. Increasing degree of substitution increased recovery of soluble complexes, while total recovery remained unchanged, and the β-amylase treatment further increased soluble complex recovery. Insoluble complexes comprised greater naringenin contents (3.91-15.15 mg/g) compared to soluble counterparts (2.45-9.43 mg/g). All complexes exhibited a mixture of B+V X-ray diffraction pattern. Overall both hydroxypropylation and β-amylase treatment improved complexation of potato starch and Hylon VII with naringenin.

KEYWORDS: hydroxypropylated starch, naringenin, β-amylase, inclusion complex, soluble complex, X-ray diffraction.

4.2 INTRODUCTION

The formation of starch inclusion complexes is mainly attributed to amylose that is capable of adopting a single left-handed helical conformation to complex with hydrophobic molecules.[1] The extent of complexation is primarily affected by amylose degree of polymerization (DP) and guest structure, which has been extensively researched using fatty acids as model compounds.[2-8] Rutschmann et al.[9] reported that the thermal stability of amylose and menthone complex increased with an increase in amylose DP. Godet et al.[10] prepared amylose of different DPs (20, 30, 40, 100 and 900 anhydroglucose units) to form complexes with caprylic, lauric, and palmitic acid, and found that as the amylose DP increased the melting temperature of the resulting complexes increased. Later, Godet et al.[11] demonstrated that amylose of DP 20 was too short to form complex with fatty acids, and the yield of complexes increased with increasing amylose DP. Recently, Arijaje et al.[12-14] modified potato starch with acetylation followed by debranching and β-amylase hydrolysis and found that linear starch chains of DPs ~50-80 was involved in the formation of complexes with stearic, oleic, and linoleic acids. Wulff and Kubik[15] first reported the formation of soluble complexes of sodium dodecyl sulfate with potato amylose of hydroxypropylation at a degree of substitution (DS) 0.075 and DPs ~9-250. Arijaje et al.[12] and Arijaje and Wang[13, 14] also showed the formation soluble complexes, and soluble complex was increased by 154-245% for stearic acid, 233-375% for oleic acid, and 327-490% for linoleic acid when potato starch was acetylated and then reduced in DP by debranching and β-amylase hydrolysis.

The complexation between starch and bioactive compounds is not well researched. The complex of starch with tea polyphenols (TPLs) was suggested to contribute to changes in starch rheological properties as well as *in vitro* starch digestion rate when TPLs were mixed with

common corn, waxy maize, and high amylose corn starches.[16-18] Different amounts of quercetin were complexed with ungelatinized and pregelatinized common corn starch, and the thermal stability of the resulting complex was governed by the quercetin content in the complex.[19] Lorentz et al.[20] studied the inclusion complex of potato amylose with chlorogenic acid and concluded that grafting 4-*O*-palmitoyl to the acid increased the complex formation. Genistein, the major isoflavone in soybean, was complexed to a greater extent with potato amylose (DP ~900) than with high amylose corn starch, implying that amylopectin long branches was also involved in the complexation.[21] Van Hung et al.[22] showed that debranched cassava starch complexed with ferulic acid, resulting in insoluble complexes of B-type X-ray diffraction pattern with improved solubility and antioxidant capacity. Recently, β-carotene exhibited improved stability and water solubility after complexed with common corn starch.[23]

Naringenin is a phenolic compound belonging to the flavanone class, and its three-ring structure effects a low water solubility.[24] Although naringenin has been shown to display anti-cancer[25], anti-inflammatory[26], and anti-microbial activities[27], the low water solubility limits its pharmaceutical application. The solubility of naringenin was improved by 10, 365, and 400-fold when complexed with β-cyclodextrin (β-CD)[28], dimethyl β-CD[29], and hydroxypropyl-β-CD[30], respectively. However, concerns remain on the toxicity and high cost of CD derivatives. In our previous study[31], potato and high amylose corn (Hylon VII) starches were acetylated and then debranched without or with DP reduction by β-amylase before complexing with naringenin. The results showed that both soluble and insoluble starch-naringenin complexes were formed and the naringenin content in both complexes increased as a result of acetylation and the enzymatic treatment. The present study followed a similar approach but using hydroxypropylation because the reported improved solubility of naringenin by hydroxypropyl-β-CD.[30,32] Therefore, the

objective of this study was to investigate the complexation of naringenin with starch that was hydroxypropylated and debranched without and with β-amylase hydrolysis, and the resultant soluble and insoluble complexes were recovered and characterized for their physicochemical properties.

4.3 MATERIALS AND METHODS

Materials

Potato starch and high amylose corn starch (~70 % amylose, Hylon VII) were kindly provided by Ingredion (Bridgewater, NJ). Potato starch was used without further treatment, and Hylon VII was defatted by extraction with 85% (v/v) methanol for 24 h, dried at 40°C for 24 h, and milled using a UDY Cyclone Mill (UDY Corporation, Ft. Collins, CO) fitted with a 0.5-mm sieve. Isoamylase (specific activity 240 U/mg), pullulanase (specific activity 34 U/mg), and β-amylase from *Bacillus cereus* (specific activity 2,182 U/mg) were purchased from Megazyme Ltd. (Wicklow, Ireland). Naringenin was obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals and reagents were of analytical grade.

Hydroxypropylation of Starch

Hydroxypropylation of potato starch and Hylon VII were carried out as described by Wang and Wang.[33] Starch (100 g, db) was used to prepare a 35% (w/w) slurry in a 1-L reaction vessel and added with Na_2SO_4 (15%, starch db). Hydration was allowed for 30 min with stirring prior to pH adjustment to 11.5 with 1 M NaOH. The vessel was carefully sealed, 4 (low) or 8% (high) propylene oxide (starch db) was added through an opening, and then the temperature was gradually increased to 45°C. After 18 h of reaction, the pH was adjusted to 5.5 with 0.1 M HCl, and the slurry was washed three times with two-fold volume of deionized water, vacuum filtered, and dried at 40°C for 24 h. The hydroxypropyl content was determined by following the

colorimetric method of Johnson[34], and the corresponding DS was calculated according to Wurzburg.[35]

Debranching of Starch

The debranching of native and hydroxypropylated starches followed the procedure of Arijaje et al.[12] with modifications. A mixture of 7.5 g starch (wet basis) and 200 mL of deionized water (3.75% w/w) was gelatinized in boiling water for 1 h. The solution temperature was equilibrated to 40°C and adjusted to pH 5.0 with 0.5 M HCl. A mixture of isoamylase and pullulanase (1.33% each, starch db) was added, and the reaction was carried at 40°C for 48 h with constant stirring. The resulting debranched starch was recovered by precipitation with pure ethanol (four-fold volume), centrifuged at 7000 ×g for 15 min, dried at 40°C for 48 h, and milled to powder using the UDY Cyclone Mill. The additional β-Amylase treatment followed the method described by Arijaje et al.[12]

Preparation of Starch-Naringenin Complex

Starch-naringenin complexes were prepared by following the method described by Arijaje et al.[12] with modifications. The pH of the debranched starch slurry (3.75% w/v) was adjusted to pH 7.0, and the temperature was gradually increased from 40 to 80°C. Naringenin (20% of starch db) that was previously dissolved in warm ethanol (6% w/v) was slowly added into the starch slurry. After 30 min at 80°C, the temperature of the mixture was adjusted to and maintained at 45°C with constant stirring for 24 h. The resulting solution was centrifuged at 7,000 ×g for 15 min to obtain the precipitate as the insoluble complex, whereas the soluble complex remained in the supernatant. Both the supernatant and the precipitate, i.e. the soluble and insoluble complexes, respectively, were dried at 40°C for 48 h and then ground using the UDY Cyclone Mill. Uncomplexed naringenin was removed from the soluble and insoluble

complexes by rinsing the complexes with 95% ethanol (10 % w/v). The mixture was vortexed for 30 s and then rotated using a labquake shaker (Barnstead/ thermolyne, Dubuque, IA) for 15 min. The slurry was centrifuged at 20,000 ×g for 10 min, and then the supernatant was descarted. A second wash was carried out under the same conditions to ensure a complete removal of uncomplexed naringenin. The resulting precipitate was dried at 40°C overnight and ground using mortar and pestle. The complex recovery was expressed as the complex weight recovered over the initial material weight.

Characterization of Inclusion Complexes

Starch Molecular-Size Distribution

The molecular-size distribution of the enzyme-treated starches was characterized by high-performance size-exclusion chromatography (HPSEC) according to Arijaje et al.[12] Ten mg of starch were solubilized in 5.0 mL of 90% dimethyl sulfoxide (DMSO), boiled for 1 h, stirred overnight at room temperature, and centrifuged at 9,300 ×g for 10 min prior to injection into a Waters HPSEC system (Waters Corp., Milford, MA). The HPSEC system was equipped with an inline degasser, a model 515 HPLC pump with a 200-µL injector valve (model 7725i, Rheodyne, Cotati, CA), a model 2414 refractive index detector, a guard column (OHpak SB-G, 6.0 × 50 mm i.d × length), and two Shodex columns (OHpak SB-804 HQ and KB-802, 8.0 × 300 mm i.d × length). The flow rate was set at 0.5 mL/min, the mobile phase consisted of 0.1 M sodium nitrate with 0.02% sodium azide, and the columns temperature was kept at 55°C and the detector at 40°C. The data was collected with an Empower Pro2 software (Waters Corp., Milford, MA), and the molecular size distribution calculated by comparing against standards of molecular weight 180.16, 828.72, 1,153, 5,200, 148,000, 872,300, and 1,100,000 g/mol (Waters Corp., Milford, MA), and 1,100,000 g/mol (Sigma-Aldrich, St. Louis, MO).

Naringenin Content in the Complex

Naringenin was released from the complex by acid hydrolysis. Twenty-five mg of the complex was added with 2.5 mL of 1 M HCl, heated in boiling water for 1 h, added with 12.5 mL of methanol, and then rotated overnight with the labquake shaker. An aliquot of 1.5 mL was transferred to a 2-mL micro-centrifuge tube and centrifuged at 9,300 ×g for 10 min. From the resulting supernatant, an aliquot of 0.5 mL was transferred to 1.5 mL screw-capped glass vials and placed in an autosampler before injection into an HPLC system (Beckman-Coulter, Fullerton, CA). The reverse-phase HPLC system consisted of an autosampler (Model 508), a dual pump (model 126), and a photodiode array detector (Model 168) and a Phenomenex (Torrance, CA) Kinetex XB-C18, 100A, 2.6 micron (100 × 4.6 mm) column. The mobile phase was a binary gradient consisting 60% 0.1% trifluoroacetic acid in water as the mobile phase A and 40% acetonitrile as mobile phase B, and run under an isocratic condition at a flow rate of 1 mL/min. A commercial standard (Sigma-Aldrich) was used as the reference to identify and quantify naringenin by comparing with the retention time. The data was collected using Beckman-Coulter System 32 Karat software (Version 8, 2006), and the results were expressed as mg of naringenin per g of the complex.

Physicochemical Properties

***Thermal Properties*.** The thermal properties of the complexes were analyzed using a differential scanning calorimeter (DSC, Diamond, Perkin-Elmer, Shelton, CT). Approximately 3 mg of the complex was weighed into a stainless steel pan, and then 10 µL of distilled water was added. The pan was hermetically sealed and allowed to equilibrate at room temperature for 18 h before scanning from 25 to 200°C, cooling from 200 to 25°C, and re-scanning from 25°C to

200°C. The scanning speed was set at 10°C/min. The onset temperature, peak temperature, end temperature, and enthalpy of any transition were calculated by Pyris data analysis software.

Wide Angle X-ray Diffraction Pattern. The X-ray diffraction pattern of the complexes was determined using a Philips PW 1830 MPD diffractometer (Almelo, the Netherlands). The X-ray generator was set at 45 kV and the current tube at 40 mA. The scanning 2θ angle went from 5° to 35° with a step size of 0.02° at 1 s per step.

Attenuated Total Reflectance Fourier Transform Infrared (ATR FT-IR) Spectroscopy. The ATR FT-IR spectra of the complexes was recorded after scanning with a Nicolet 8700 spectrometer (Thermo Electron Scientific Inc., Waltham, MA) using a Golden Gate ATR accessory (Specac) equipped with a single-reflection diamond crystal. A constant temperature (25°C ± 0.1°C) was maintained during the scanning, and for each sample, 64 scans were collected with a resolution of 4 cm^{-1}. The spectrometer's EverGlo source was on turbo mode during measurements. The complexes were conditioned for 7 days in a desiccator containing silica gel prior to analysis.

Statistical Analysis

The experiment was replicated two times. For each analysis, at least two duplicates were conducted. The data was analyzed using JMP Pro13 Software (SAS Institute Inc., Cary, NC, USA), and the means compared using Tukey's honestly significant differences (HDS) test.

4.4 RESULTS AND DISCUSSION

4.4.1 Molecular Size Distribution

Figure 4.1 illustrates the molecular size distribution of native and hydroxypropylated (HP) potato starch and Hylon VII that were debranched or debranched/β-amylase treated. In general, debranched potato and Hylon VII starches displayed four and three peaks, respectively, and three and two peaks, respectively, when the β-amylase treatment was incorporated. Hydroxypropylation resulted in an increase in the proportion of large molecular size peaks, which was ascribed to an increase in hydrodynamic volume from the substituted hydroxypropyl groups. When the β-amylase treatment was included, the molecular size distributions became narrower and peak DP of all peaks shifted to shorter retention times. A similar trend was observed in our previous study with acetylated starches.[31] Hylon VII starches with or without β-amylase hydrolysis displayed a larger proportion of longer DP chains ~40-950 (Figure 4.1B and 4.1D); potato starches showed a larger proportion of shorter DP chains ~40-140 (Figure 4.1A and 4.1C) after the same treatments. For both starches the fraction of peak DP 35-45 decreased with increasing hydroxypropylation level.

4.4.2 Degree of Substitution (DS)

The initial DS values of low and high HP DS of potato starch were 0.051 and 0.129, respectively, and those of low and high HP Hylon VII were 0.063 and 0.122, respectively. Table 4.1 summarizes the DS values of soluble and insoluble complexes of potato starch and Hylon VII that were debranched or debranched/β-amylase treated. For the same treatment, soluble complex displayed a similar or greater DS compared to that of insoluble complex, agreeing with our previous study of acetylated potato and Hylon VII starches.[31] The DS values of high HP potato starch complexes were significantly greater than those of high HP Hylon VII, which was

attributed to the presence of a greater proportion of shorter chains in potato starch. The incorporation of the β-amylase treatment did not alter the DS for both complexes of both starches. The trend was different from our previous study with acetylated starches, where the DS of all complexes increased with the introduction of β-amylase treatment. It is hypothesized that acetyl groups were predominantly introduced in the amorphous lamella close to the branching points, whereas the hydroxypropyl groups may be present in the amorphous lamella close to the branching points as well as the amorphous regions close to the non-reducing ends due to their different reaction condtions.[33,36-38] Because β-amylase cannot bypass the hydroxypropyl groups in the non-reducing ends, the resultant starch chains comprised a greater amount of hydroxypropyl groups compared with acetylated starch in the previous study.[31]

4.4.3 Complex Recovery and Naringenin Content

Table 4.1 presents the recovery, which was expressed as recovered complex weight (g) over initial materials weight (g), and the naringenin content in both soluble and insoluble complexes for all treatments. The total recovery ranged 0.91-0.99 g/g and similarly to previous studies[12-14,31] the combination of hydroxypropylation with the enzymatic treatment had no significant impact on the total recovery of both complexes. Hydroxypropylation significantly increased the formation of soluble complex, whereas the β-amylase treatment did not change the recovery of soluble or insoluble complex in both starches.

For the same treatment, the naringenin content was greater in the insoluble complex than in the soluble complex, and was greater in Hylon VII complexes (6.72-15.15mg/g) than in potato starch complexes (2.45-11.18 mg/g), agreeing with our previous study[31] of acetylated Hylon VII and potato starch. The naringenin content generally increased with increasing DS of HP starches, and increased for low HP starches when the β-amylase treatment was incorporated for both

complexes. The bulky hydroxypropyl groups at a high hydroxypropylation level may interfere with the conformation of helical structure, thus resulting in reduced complexation capability. Furthermore, hydroxypropyl groups are less hydrophobic than acetyl groups. Thereby, it is suggested that a combination of decreased hydrophobicity and increased stearic hindrance was responsible for the lower complexation yield of HP starches compared with acetylated starches.[31,39,40] Wulff showed that the soluble complex of HP potato amylose (DS 0.13) complexed a greater amount of iodine than that of acetylated counterpart of a similar DS (0.14).[32] The insoluble complex of acetylated Hylon VII was reported to comprise more α-naphthol than that of HP Hylon VII.[41] These discrepancies suggest that guest molecule structure and DS of starch also impacted complexation yield besides substitution type.

Starch DP involved in complexing with fatty acids was suggested to be greater than 20 but less than 400.[42-44] Starch chains greater than DP 400 were believed to be too long to form an ordered helical structure, but starch chains less than DP 20 were too short to induce helical structure. When considering all treatments, the majority of starch chains were present approximately between DP 40 and 200 for potato starch and between 40 and 600 for Hylon VII (Figure 4.1). Because of a greater naringenin content in Hylon VII complexes than in potato starch complexes, starch chains longer than DP 200 were suggested to be also involved in complexing with naringenin, similar to the DP ranges found in acetylated starches.[31]

The ferulic acid in the complex with debranched cassava starch ranged 6.8-31.5 mg/g[23]; the genistein content in the complex with unmodified potato amylose ranged 14-113 mg/g.[21] It should be noted that both studies only reported the bioactive compounds in the insoluble complex. The greatest complexed naringenin contents in the insoluble complexes were 11.18 and 15.15 mg/g for potato starch and Hylon VII, respectively, and the naringenin contents in the

soluble complexes ranged 2.45-9.43 mg/g in the present study. The differences among these studies confirm that the structure of the guest molecule was of great importance in determining the extent of complexation. The non-planar conformation of naringenin B-ring compared with genistein is suggested to contribute to the lower complexation of naringenin since changes on its orientation might prevent naringenin from properly positioning inside the helical structure, thus forming unstable complexes.

4.4.4 Physicochemical Properties of Starch-Naringenin Complexes

Wide Angel X-ray Diffraction Pattern

Figures 4.2 and 4.3 display the X-ray diffraction pattern of both complexes from potato starch and Hylon VII, respectively. For all soluble and insoluble complexes, hydroxypropylation resulted in an increased amorphous structure compared to the native counterpart. The V-type X-ray diffraction pattern, with a weak diffraction angle 2θ at 19.9°, was greatly affected by hydroxypropyl groups that might prevent the alignment and formation of the helices for a more defined V-type structure. Unlike acetylated potato starches[31], the β-amylase treatment did not increase the V-type structure but generally increased the amorphous structure. In contrast the β-amylase treatment slightly decreased the amorphous structure of Hylon VII complexes, which was attributed to an increase in the proportion of longer DP chains (Figure 4.1).

Except for the soluble complex of native potato starch displaying the A-type pattern, all soluble and insoluble complexes showed a mixture of B+V type crystalline structure. Most complexes of acetylated potato and Hylon VII also showed a mixture of B+V patterns, except the soluble complexes of all treated potato starch displaying A+V-type. Zhang et al.[19] studied the complexation of lauric acid with debranched high amylose corn starch (Hylon V), and observed that as the debranching time increased from 0 to 24 h, the X-ray diffraction pattern of the

insoluble complexes shifted from the V-type to a mixture of B+V-type.[19] Van Hung et al.[23] only observed the B-type crystalline structure for the insoluble complex of ferulic acid with debranched cassava starch.[23] These results indicate the importance of starch DP and the introduction of substituents on the structure of the resultant complexes.

Attenuated total reflectance Fourier Transform Infrared (ATR FT-IR) Spectroscopy

The ATR FT-IR spectra of soluble and insoluble complexes along with the physical mixture of naringenin with potato starch and Hylon VII are shown in Figures 4.4 and 4.5, respectively. Forrest and Cove[45] suggested that the absorption of methyl group of hydroxypropyl substituents occurred between ~2850-3000 cm^{-1} (CH-stretching) and ~1350-1475 cm^{-1} (CH deformation), which is the same range where native starch naturally present groups, i.e. C-O-C, display strong absorption, hence the characteristic bands overlap. The FT-IR spectra of HP starches and their complexes displayed an increase in the intensity of the bands at ~2850-3000 cm^{-1} and ~1350-1475 cm^{-1} with increasing DS of hydroxypropylation, which was attributed to the incorporation of hydroxypropyl groups into starch chains. Although no appreciable difference was observed between the spectra of parent starches and that of the complexes, the spectra of the physical mixture was different and displayed a superimposition of the individual spectra of starch and naringenin.

Analysis of the differential spectra (data not shown) revealed that the bands ~2850-3000 cm^{-1} and ~1350-1475 cm^{-1} remained for all the complexes, and therefore it is suggested that hydroxypropyl groups were involved in the complex. The intensity of the band was greater for the soluble complexes than the insoluble complexes, which is agreement with the DS results (Table 4.1) where soluble complexes generally had a greater DS. In agreement with our previous study with acetylated starches[31] the bands between ~3600-3000 cm^{-1} (hydroxyl groups)

were present after the subtraction of the spectrum of starch and naringenin, suggesting that some molecular interaction between naringenin and starch was involved in the complex formation. It should be noted that the intensity of the bands between ~3600-3000 cm^{-1} was greater for Hylon VII than for potato starch, confirming a greater involvement of Hylon VII chains in complexing with naringenin. The differential spectrum (data not shown) also showed that the band ~988 cm^{-1} (CO stretching of ring B)[46] was involved in both complexes, indicating that the B ring of naringenin was involved in complexing with starch chains. When the β-amylase treatment was incorporated, a slight increase in the band ~988 cm^{-1} was observed, which might be related to the amount of naringenin complexed. This effect was more evident in HylonVII, presumably because of its greater complexing capability. These results corroborate the X-ray diffraction pattern results and also confirm that some molecular interaction took place between starch and naringenin in forming complexes of different crystalline structures instead of physical mixtures.

Thermal Properties by Differential Scanning Calorimetry

The complexes from native starches displayed a typical endothermic transition at the first scan; however, the complexes from HP starches exhibited two exothermic peaks during the first scan and a single endothermic peak at the second scan, which was not observed in our previous study[31] of acetylated starches. The bulky hydroxypropyl groups may effect a less organized packing of the helices[38,40,47], which rearranged to become more ordered during the first scan. The soluble complexes did not exhibit any thermal transition, whereas the insoluble complexes displayed a transition between 100.7 and 122.2 °C (Table 4.2) for both native starches.

When HP starches were used, the onset melting temperature decreased, the melting temperature range reduced, and the enthalpy decreased, except that the melting temperature range increased for high HP potato starch. The high enthalpy values of complexes from native

starches relative to other complexes indicate the role of hydroxypropyl groups in destabilizing the complexes. The complexes of high HP potato starches exhibited the lowest melting temperatures and a large melting temperature range similar to those of native starches. For Hylon VII, hydroxypropylation resulted in decreased melting temperatures and enthalpy, but melting temperature range remained unchanged with a further increase in DS. It is proposed that the crystalline structure in high HP potato starch complexes comprised a mixture of crystallites with varied thermal stability due to the presence of less homogeneous DP distribution compared with Hylon VII, which produced complexes of overlapping endotherms. The β-amylase treatment increased melting temperature of some complexes of HP potato starch, but decreased melting temperatures of some complexes of HP Hylon VII.

In conclusion, hydroxypropylation and the β-amylase treatment resulted in larger proportions of starch chains of longer DP for both Hylon VII and potato starch and increased complexed naringenin contents, suggesting that longer DP chains were involved in complexing with naringenin. HP starches formed both soluble and insoluble complexes with naringenin, and the introduction of hydroxypropyl groups enhanced the recovery of the soluble complexes. The naringenin content was greater in the insoluble complex than in the soluble complexes, and generally increased with increasing DS of hydroxypropylation. When the β-amylase treatment was included, a further increase in naringenin content was observed for low HP starches. FT-IR results confirmed the occurrence of molecular interaction between starch and naringenin in both complexes. A combination of favored DP range and hydroxypropylation level of starch was important in maximizing its complexation with naringenin.

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Table 4.1. Degree of Substitution, Complex Recovery and Percentage of Naringenin Content Recovered from All Complexes[a]

debranched starch	hydroxypropylation	β-amylase treatment	type of complex	DS of hydroxypropylation	recovery (g/g)	naringenin in individual complex (mg/g)
potato	no	no	soluble	NA[b]	0.30±0.01C	2.45±0.01D
		no	insoluble	NA	0.64±0.00b	4.42±0.11g
		yes	soluble	NA	0.33±0.02C	2.67±0.33D
		yes	insoluble	NA	0.62±0.01b	3.91±0.25g
	low	no	soluble	0.063±0.001C	0.60±0.01B	4.35±0.40CD
		no	insoluble	0.047±0.000d	0.32±0.00d	5.34±0.28fg
		yes	soluble	0.068±0.002C	0.65±0.01B	5.09±0.47B-D
		yes	insoluble	0.060±0.000cd	0.29±0.01d	11.18±2.15b-d
	high	no	soluble	0.153±0.000A	0.74±0.02A	6.75±2.77A-C
		no	insoluble	0.150±0.002a	0.19±0.01e	9.29±0.04c-e
		yes	soluble	0.154±0.001A	0.73±0.04A	4.34±0.75CD
		yes	insoluble	0.151±0.002a	0.18±0.00e	7.73±0.55ef
Hylon VII	no	no	soluble	NA	0.12±0.00D	6.72±1.12A-C
		no	insoluble	NA	0.87±0.00a	8.06±1.20d-f
		yes	soluble	NA	0.14±0.00D	8.82±0.07AB
		yes	insoluble	NA	0.83±0.00a	13.61±0.52a-b
	low	no	soluble	0.066±0.000C	0.65±0.04B	6.87±0.51A-C
		no	insoluble	0.067±0.000c	0.31±0.03d	11.33±0.10bc
		yes	soluble	0.066±0.004C	0.60±0.00B	7.73±0.02A-C
		yes	insoluble	0.059±0.001cd	0.37±0.00c	15.15±0.80a
	high	no	soluble	0.115±0.008B	0.79±0.01A	9.43±0.41A
		no	insoluble	0.092±0.001b	0.18±0.00e	10.97±0.17b-d
		yes	soluble	0.107±0.008B	0.78±0.01A	9.21±1.15A
		yes	insoluble	0.082±0.008b	0.18±0.00e	10.31±0.04c-e

[a]Mean of two replicates with standard deviation. Mean values in the same column followed by different uppercase or lowercase letters are significantly different based on Tukey's HSD test. [b]NA: Not applicable.

Table 4.2. Melting Temperatures and Enthalpies[a] of Recovered Soluble and Insoluble Fractions of Native and Hydroypropylated Potato Starch and Hylon VII Naringenin Complexes.

debranched starch	hydroxypropylation	β-amylase treatment	type of complex	Temperature (°C)			Enthalpy (J/g)
				onset	peak	end	
potato	no	no	soluble	ND[b]	ND	ND	ND
		no	insoluble	100.7±0.2a	110.1±0.9b	118.3±0.20b	8.9±0.2a
		yes	soluble	ND	ND	ND	ND
		yes	insoluble	101.0±0.2a	109.7±0.1b	118.1±0.02b	9.7±0.2a
	low	no	soluble	84.0±0.1B	86.5±0.1CD	91.9±0.3B-D	2.6±0.1A-C
		no	insoluble	83.1±0.1c	88.9±0.0cd	93.6±0.1d	2.6±0.1cd
		yes	soluble	85.5±0.0A	91.1±0.2A	97.1±0.1A	2.5±0.5A-C
		yes	insoluble	85.1±0.3b	90.7±0.3c	96.7±0.3c	2.3±0.3cd
	high	no	soluble	76.7±0.1F	88.5±0.5B	94.6±0.6AB	1.6±0.5CD
		no	insoluble	76.9±0.2d	87.4±0.7de	93.4±0.4d	2.5±0.5cd
		yes	soluble	77.5±0.5E	88.6±0.0B	94.0±0.1A-C	1.7±0.0B-D
		yes	insoluble	76.9±0.2d	87.4±0.7de	93.4±0.4d	2.5±0.5cd
Hylon VII	no	no	soluble	ND	ND	ND	ND
		no	insoluble	101.8±0.9a	112.5±0.5a	122.2±0.8a	4.1±1.0bc
		yes	soluble	ND	ND	ND	ND
		yes	insoluble	102.2±0.9a	114.4±0.3ab	119.7±0.9b	5.4±0.9b
	low	no	soluble	82.7±0.2C	85.5±0.0DE	91.3±2.0CD	2.9±0.4AB
		no	insoluble	82.7±0.2c	85.6±0.1ef	92.6±0.1de	2.6±0.6cd
		yes	soluble	81.8±0.2D	84.4±0.4E	87.8±0.2E	3.0±0.2A
		yes	insoluble	82.7±0.0c	86.0±0.7ef	92.6±0.5de	2.5±0.2cd
	high	no	soluble	82.3±0.1CD	86.9±0.4C	90.3±0.7DE	2.5±0.0A-C
		no	insoluble	82.3±0.5c	85.2±0.1f	91.8±0.1de	1.6±0.2d
		yes	soluble	83.0±0.0C	86.0±0.4CD	91.2±0.7CD	1.2±0.0D
		yes	insoluble	82.8±0.4c	85.9±0.1ef	90.7±1.6e	1.4±0.2d

[a]Mean of at least two replicates with standard deviation. Mean values in the same column followed by different uppercase or lowercase letters are significantly different based on Tukey's HSD test. [b]ND: not detected.

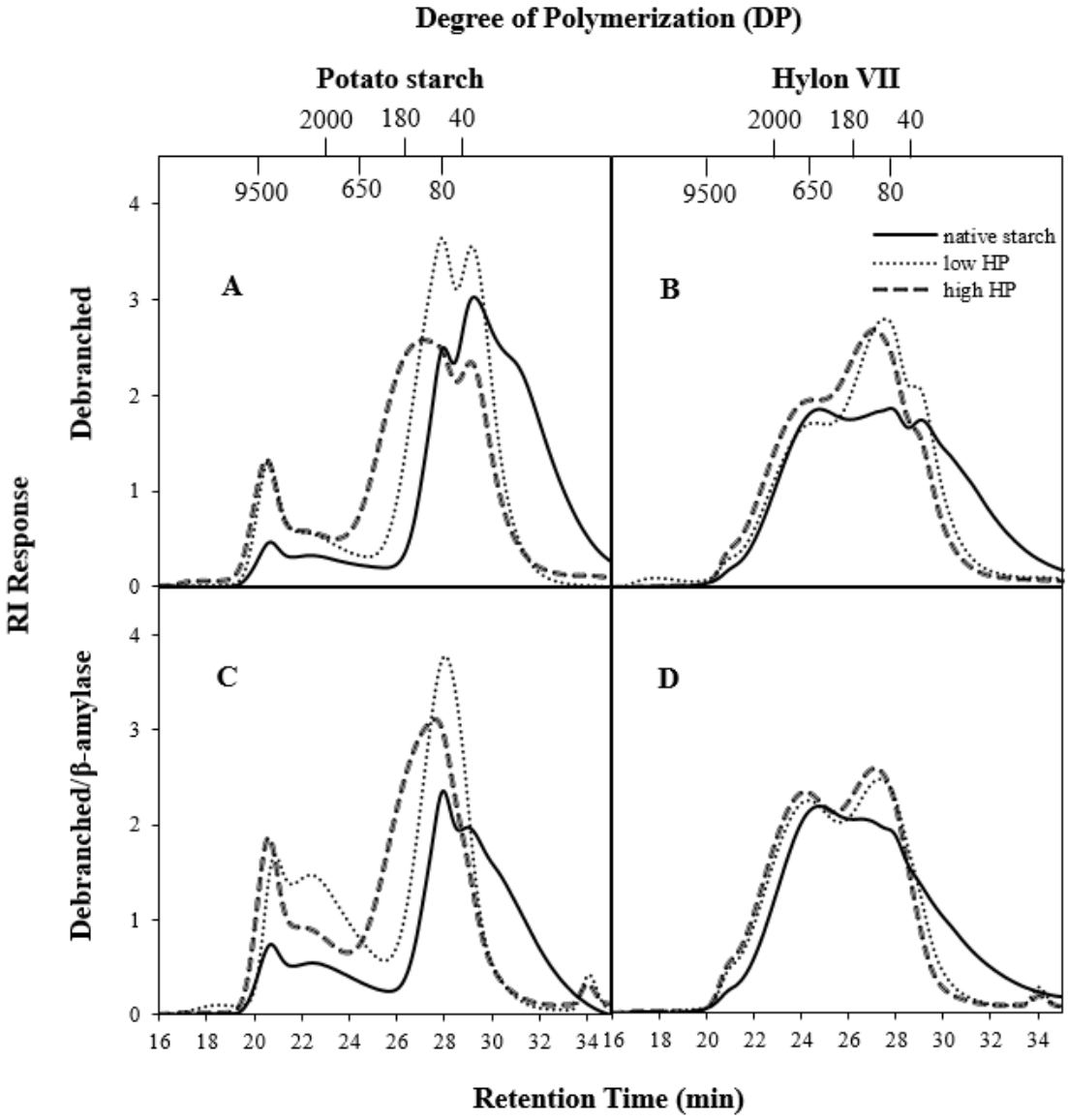

Figure 4.1. Normalized size-exclusion chromatograms of native and hydroxypropylated (HP) debranched and debranched/β-amylase treated potato and Hylon VII starches.

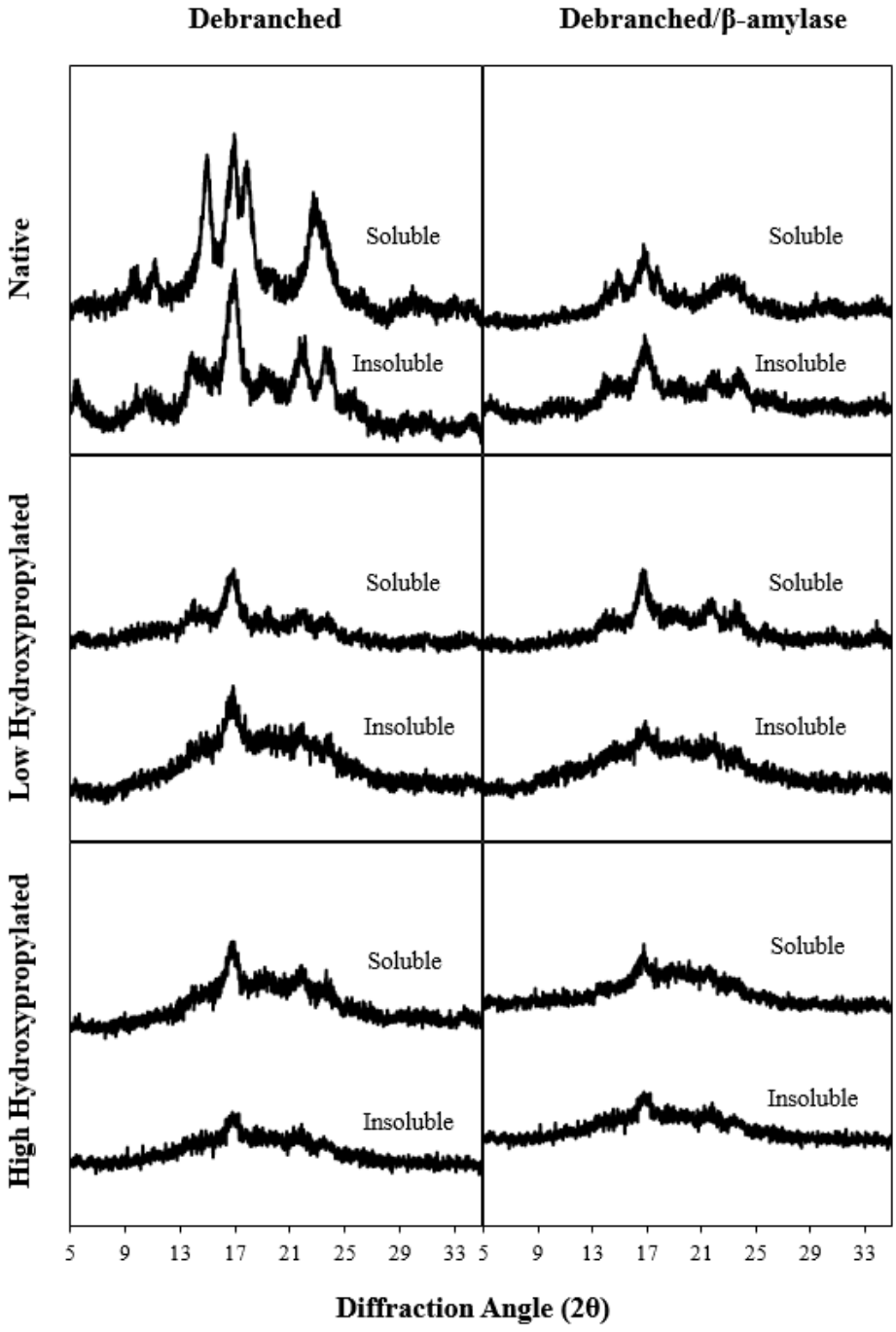

Figure 4.2. X-ray diffractograms of soluble and insoluble starch-naringenin complexes of native and hydroxypropylated debranched and debranched/β-amylase treated potato starch.

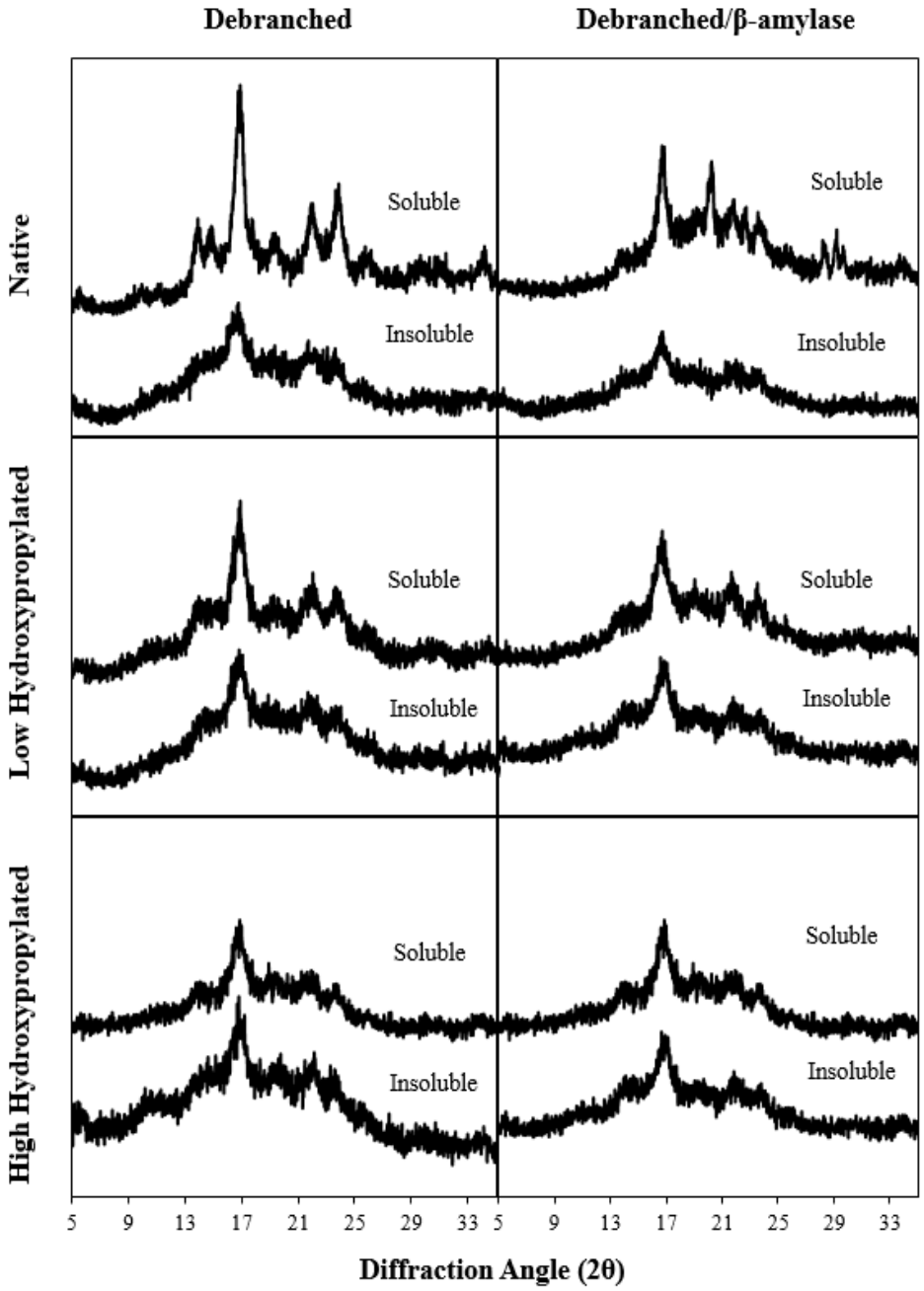

Figure 4.3. X-ray diffractograms of soluble and insoluble starch-naringenin complexes of native and hydroxypropylated debranched and debranched/β-amylase treated Hylon VII.

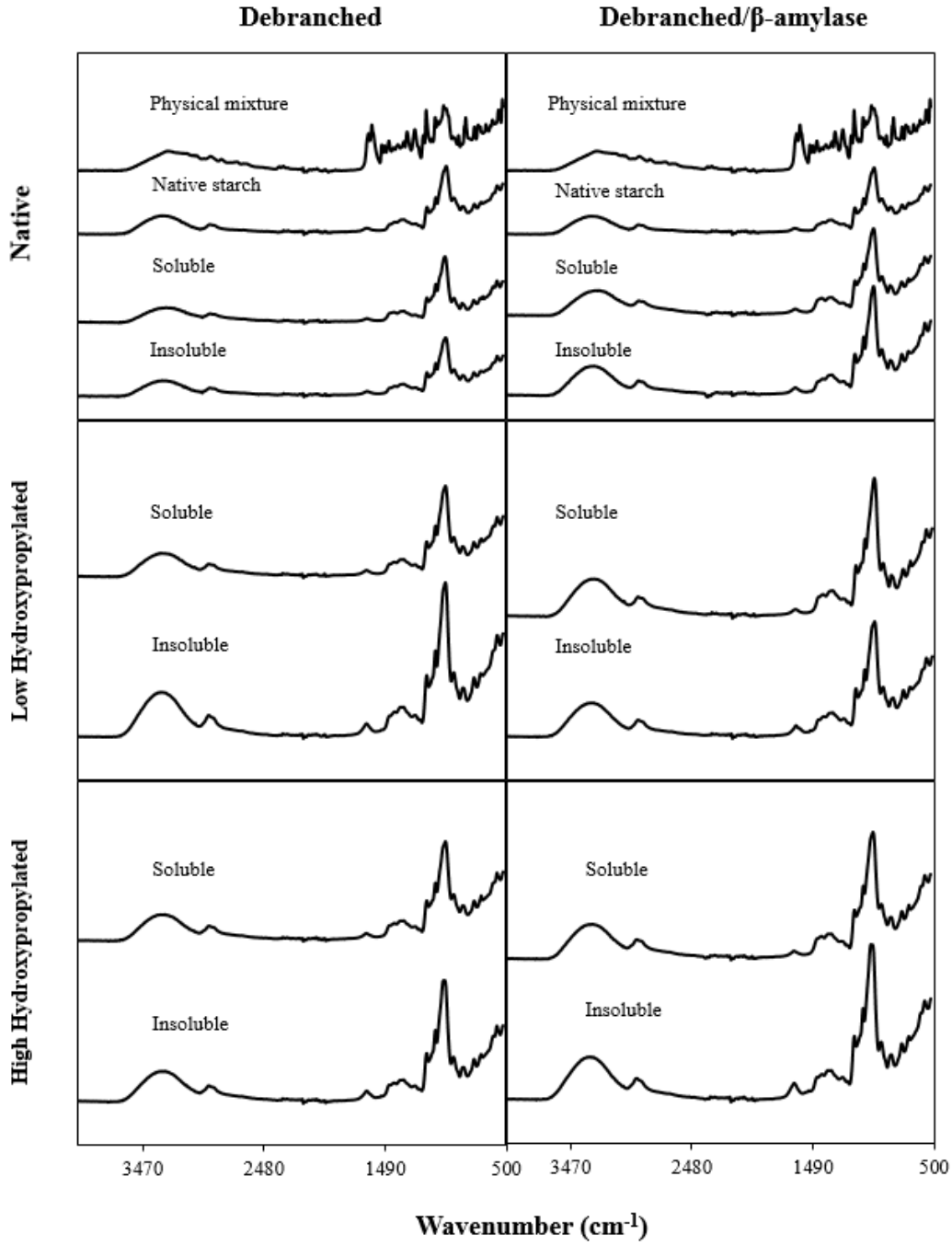

Figure 4.4. Attenuated Total reflectance Fourier Transform infrared (ATR FT-IR) spectra of soluble and insoluble starch-naringenin complexes of native and hydroxypropylated debranched and debranched/β-amylase treated potato starch. The spectra of native potato starch and physical mixture of native potato starch and naringenin are included for comparison.

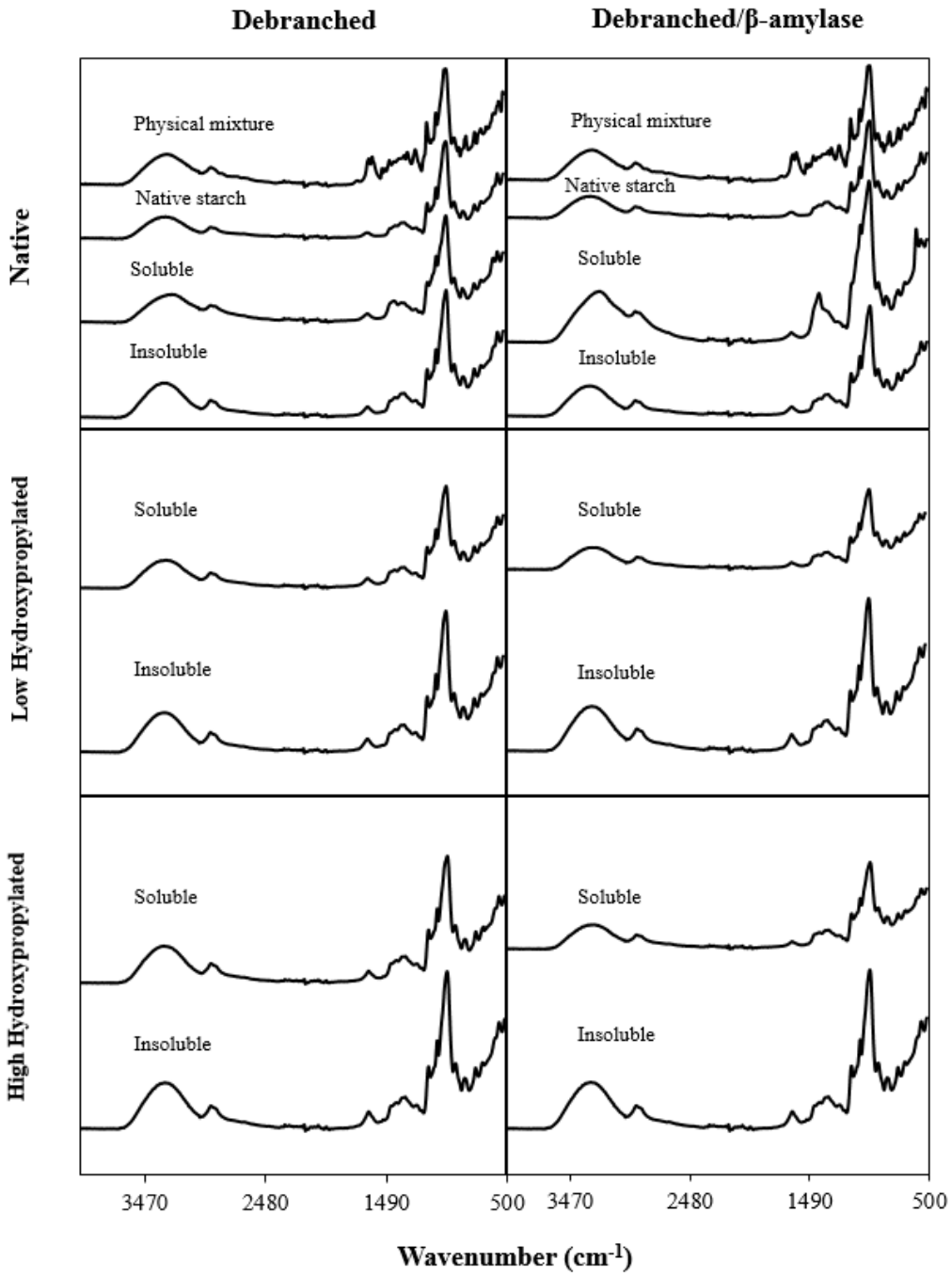

Figure 4.5. Attenuated Total Reflectance Fourier Transform Infrared (ATR FT-IR) spectra of soluble and insoluble starch-naringenin complexes of native and hydroxypropylated debranched and debranched/β-amylase treated Hylon VII. The spectra of native Hylon VII and physical mixture of native potato starch and naringenin are included for comparison.

CHAPTER 5

OVERALL CONCLUSION

This study demonstrated that naringenin formed both soluble and insoluble complexes with starch, and Hylon VII complexes comprised greater naringenin contents than potato starch complexes. The introduction of substituents and β-amylase treatment increased the recovery of soluble complexes and generally increased complexed naringenin content. The β-amylase treatment resulted in an increase in the fraction of starch chains with longer DP for both potato and Hylon VII starches, and starch chains of DP >400 were involved in complexation. HP starches exhibited reduced complexation with naringenin compared with acetylated starches because of the high DS and bulky hydroxypropyl groups. The X-ray diffraction pattern of both complexes was determined by the DP range of starch chains, and a mixed crystalline structure was observed for most complexes. The changes in melting behavior were attributed to a decrease in the crystalline structure and disruption of the helical structure stability by acetyl and hydroxypropyl groups. The complexation capability of starch was enhanced by acetylation and hydroxypropylation, although the differences observed suggest that there was an optimum combination of DS and DP to maximize complexation. The distribution of the substituents in starch chains was also an important factor to be considered besides the structure of naringenin.