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Structural and Functional Properties of Amylose Complexes with Genistein

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Complexes of amylose or high-amylose corn starch (HACS) with genistein were prepared by the acidification of an alkali solution to yield a V_{6III} structure. The amylose−genistein complexes exhibited significantly higher genistein content (11 mg/100 mg of complex) than HACS−genistein complexes (9 mg/100 mg of complex). The effect of genistein on the amylose complexes was examined in different genistein−amylose ratios, and a model for genistein organization in the amylose complexes was suggested. The complexes were stable at different pH values, with <10% of the complexed genistein released, and were stable at 30 and 50 °C. Lower stability was observed at 80 °C as shown by the extensive release of genistein. All complexes showed high retention of genistein in simulated stomach conditions and released genistein upon digestion in pancreatin solution. It is therefore suggested that the complexes can be used as carriers for the slow release of genistein.

KEYWORDS: Isoflavones; genistein; amylose; starch; nanoencapsulation; slow release

INTRODUCTION

Nutraceuticals and functional foods are attractive to consumers due to their ability to provide health benefits along with high acceptability. The oral bioavailability of poorly water soluble nutraceuticals, such as essential fatty acids, lipophilic vitamins, and soy isoflavones, can be increased by formulation approaches, which increase the solubility and dissolution rates (*1, 2*). There are various methods for creating oral delivery systems; the challenge is to create a low-cost system that will be acceptable to the consumer. One approach is based on hydrophobic interactions between molecules as a basis for their slow release (*3*). Amylose is known for its ability to form inclusion complexes with hydrophobic ligands (guest molecules) (*3, 4*). This can be exploited as a possible technological platform having the advantages of starch availability, low cost, and GRAS status.

Amylose chains can be organized in disordered amorphous conformation or in two types of helical forms: the double helix of the amylose chains (A or B form) or a single-helix structure (V-form), which is induced in the presence of hydrophobic guest molecule (*4–6*). Amylose complexes with guest molecules are based on the interaction between amylose and hydrophobic molecules such as iodine, fatty acids, and aromatic compounds, which form single-helix structures known as the V form (*3, 7, 8*). Predominant in the V amylose family is the common form of V_6 amylose, characterized by six glucose units per turn (*9–13*); however, for some guest molecules the helix exhibits a different structure. These guest molecules are bulkier and form a structure known as V_8. The V_8 structure exhibits a larger cavity in the helical structure, having eight glucose residues per turn, which allows the inclusion of larger molecules (*14, 15*). In the V_6 family there are three types of known structures: V_{6I}, V_{6II}, and V_{6III}, depending on the guest molecule. These structures differ in the volume existing between helices. In the V_{6I} family the guest molecule is trapped inside the helix cavity. In the V_{6II} and V_{6III} forms, the guest molecule can be trapped within or between helices (*7*). Among the molecules that induce the V_{6III} form are isopropanol, *tert*-butyl alcohol, acetone, propionic acid, 1,5-decanolactone, and linalool (*4, 7, 15*). The existence of a V_7 structure is debatable. This structure was suggested in the past to branched alcohols such as *tert*-butanol and isopropanol (*16*). However, other studies claim that these molecules induce a V_{6III} structure (*9, 17, 18*).

Although amylose has been known for its ability to create inclusion complexes for many years, data on amylose inclusion complexes with polyphenolic materials is scarce. Several studies examined amylose complexes with small aromatic molecules such as salicylic acid and its analogues (*19–21*), and others examined amylose complexes with molecules having two aromatic rings such as α-napthol (*5, 9, 11, 22, 23*).

Isoflavones are natural compounds, potentially therapeutic, that are found in a variety of plants, predominantly soybean. These are the most common phytoestrogenic compounds found in plants, known for their ability to act as partial estrogen agonists and antagonists in influencing cell biochemistry by direct and indirect actions on enzymes, growth factors, and genes (*24–26*). Isoflavones engage in biological activity associated with a variety of beneficial health effects including

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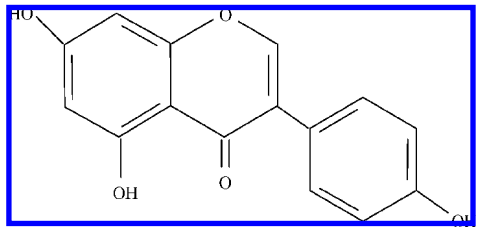

Figure 1. Genistein molecular structure.

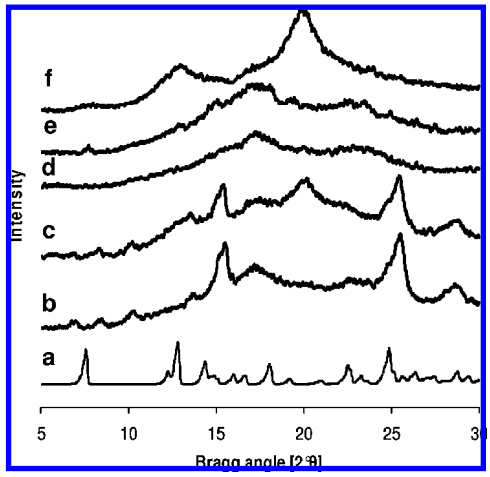

Figure 2. X-ray diffraction patterns of pure genistein (**a**), genistein complexes with amylose (**b**) and HACS (**c**), amylose without genistein (**d**), genistein−amylose physical mixture (**e**), and HACS without genistein (**f**). Amylose and HACS without genistein were used as reference.

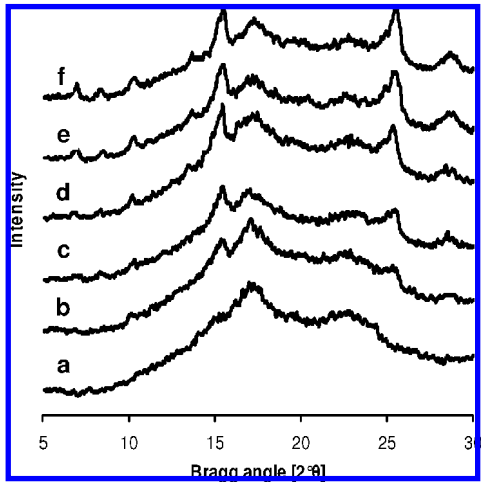

Figure 3. X-ray diffraction showing the influence of genistein amounts on complex structure tested. Six genistein−amylose ratios are presented: 1/60 (**a**), 1/30 (**b**), 1/20 (**c**), 1/15 (**d**), 1/12 (**e**), and 1/10 (**f**).

reduction of risk of cardiovascular disease, lowering rates of prostate, breast, and colon cancers, and improving bone composition (*27*). The chemical form of the isoflavones and their metabolites influences the extent of absorption. The aglycones are more readily absorbed and are more bioavailabile than conjugated spices (*28*). Because genistein, 4′,5,7-trihydroxyisoflavone (**Figure 1**) is the most abundant aglycone form of isoflavone found in soybean, it was chosen as the guest molecule in this research (*29*). The use of genistein in food and beverage is restricted due to its bitter taste (*30*) and poor aqueous solubility (*1*). The poor solubility of genistein in water also reduces its bioavailability (*1, 31*).

The goal of this study was to create a delivery system for the protection and slow release of genistein in the digestive tract. Complexes with different genistein amylose ratios were characterized by X-ray diffraction (XRD) and particle size analysis. The stability of the complexes was tested under simulated stomach and intestinal conditions and different pH levels and temperatures.

MATERIALS AND METHODS

Materials. High-amylose corn starch (HACS) was obtained from National Starch (HYLON VII; Bridgewater, NJ). Potato amylose type III (average DP 900, essentially free from amylopectin, A0512) and pancreatin (amylase, 41 USP; from porcine pancreas, P1500) were obtained from Sigma Chemical Co. (St. Louis, MO). Genistein was purchased from LC Laboratories (Woburn, MA; BNG-6055). All other reagents were of analytical grade.

Methods. *Preparation of Amylose−Genistein Complexes.* Complexation was carried out by the acidification of an alkali solution (KOH/H_3PO_4) as previously described for other compounds (*3, 4*). Amylose was dissolved in 0.1 N KOH solution (60 mL, 10 mg/mL of 0.1 N KOH, pH 12.5) at 90 °C. Genistein solution was prepared separately at 30 °C (40 mL, 1.5 mg/mL of 0.1 N KOH, pH 12.5). The solutions were mixed at 30 °C, and the mixture was precipitated by adjusting the pH to 4.7 (±0.5) by using 2% H_3PO_4 and held for 24 h under gentle stirring. All samples were then centrifuged (20000*g*, 25 min, 4 °C), the supernatant was discarded, and the precipitate was washed twice with an ethanol/water mixture (50:50 w/w) and centrifuged as before. The complexes were then freeze-dried.

HACS complexes were produced in the same way as amylose complexes. However, HACS was defatted first to remove excess of fatty acids that may be complexed and compete with genistein (*32*). HACS was defatted by Soxhlet apparatus, extracting the fatty acids with petroleum ether for 3 h, followed by vacuum-drying at 80 °C.

Characterization of the Complexes. (A) X-ray Diffraction. XRD measurements were carried out by a Philips PW 3020 powder diffractometer equipped with a graphite crystal monochromator. The operating conditions were Cu Kα1 radiation (0.154 nm), voltage 40 kV, and current 40 mA. Samples were scanned over 5−30° 2θ in steps of 0.02° 2θ per second, and the crystalline nature of the complex was determined by the position of the X-ray diffraction peaks.

(B) Particle Size Analysis. Suspensions produced by the complexation process were analyzed by laser scattering to monitor the particle size distribution of the resulting inclusion complexes. The particle size distribution was analyzed by a LS 230 Coulter Counter particle size analyzer and by dynamic light scattering (DLS). The Coulter Counter (Coulter Corp.) was equipped with a polarized intensity differential scattering module (PIDS module) using three wavelengths and two polarization directions. The laser scattering data of the suspensions was collected in two sequential periods of 90 s. The data obtained were processed using LS230 software, based on the general Fraunhofer optical model, with water as solvent, to plot particle size distribution curves and calculate mean particle size and standard deviation.

The size of particles that were too small to be detected by the Coulter Counter was analyzed by DLS. Five hundred microliters of the sample was inserted into glass vials, which were placed in a NICOMP 380ZLS particle size analyzer (Santa Barbara, CA). Data were recorded at 30 °C over a period of 10 min. Mean particle size and particle size distribution were then calculated using ZPW388 software. All of the results were reported as volume distribution assuming ideal spheres (software default).

Genistein Content. Genistein content in the complex was tested by full dissolution of the complexes in 0.1 N KOH for the release of the guest molecule. Fifteen milligrams of the complex was incubated in 1 mL of 0.1 N KOH solution at 37 °C for 24 h. Because genistein is poorly soluble in water, after this incubation, samples were diluted by phosphate buffer (PBS) to a concentration below their solubility limit (*25*). PBS (159 mL, 20 mM phosphate, pH 6.9, and 10 mM NaCl) was added to the solution, and the solution was centrifuged (5500*g*, 15 min). Genistein was quantified from the supernatant by reverse-phase high-performance liquid chromatography (RP-HPLC), HP 1100, equipped

Table 1. Effect of Genistein−Amylose Ratios on Genistein Concentration in the Complex

genistein−amylose ratio in complexation solution (w/w)	genistein content in the complex (mg of genistein/100 mg of complex)[a]	glucose−genistein molar ratio in the complex	particle size	
			DLS (nm)	Coulter counter(μm)
1/60	1.4 ± 0.3	115	127 ± 87	
1/30	2.0 ± 0.6	82		
1/20	3.0 ± 0.3	54		
1/15	5.2 ± 0.9	30		38 ± 44
1/12	10.8 ± 2.6	14		114 ± 95
1/10	11.3 ± 1.7	13		93 ± 36

[a] Calculated per 100 mg of complex [genistein content in the complex (%) = genistein in the complex (mg) × 100/amylose−genistein complex (mg)].

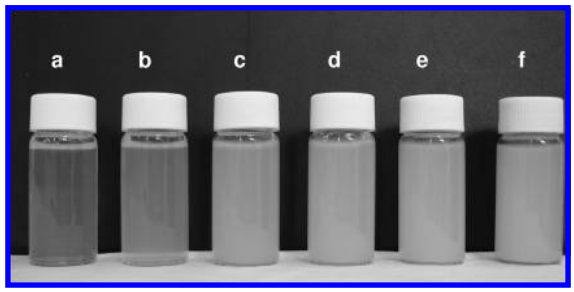

Figure 4. Image of genistein−amylose complexes at different genistein/amylose ratios: 1/60 (**a**), 1/30 (**b**), 1/20 (**c**), 1/15 (**d**), 1/12 (**e**), and 1/10 (**f**).

with a diode array detector and autosampler and controlled by ChemStation software (Hewlett-Packed, Wilmington, DE). HPLC analysis was carried out on a reverse phase C_{18} column 250 × 4 mm with 5 μm packing. Samples were eluted at a flow rate of 1 mL/min by solvent A [0.1% acetic acid in water (v/v)] and solvent B (acetonitrile). The gradient elution was from 5 to 35% B in a linear gradient over 33 min, washing with 100% B for 5 min, and then equilibrated for 10 min between runs with 5% B. The injection volume was 20 μL, and detection was done by UV absorbance at 254 nm (*33*). The amount of genistein in the complex was quantified by calibration curve using pure genistein as a standard. Genistein content in the complex was calculated per 100 mg of complex using the following equation:

$$\text{genistein content in the complex (\%)} = \frac{\text{genistein in the complex (mg)} \times 100}{\text{complex weight (mg)}} \quad (1)$$

Different Genistein−Amylose Ratios. Complexes were produced by acidification as mentioned above. A set of six samples was prepared with increasing genistein−amylose ratios: 1/60, 1/30, 1/20, 1/15, 1/12, and 1/10 (w/w). The structures of the complexes were examined by X-ray diffraction and by particle size analysis. The total genistein content was quantified by total dissolution of the complexes in KOH solution as previously described.

The release of genistein by enzymatic digestion was measured by exposing the complex to mammalian pancreatic amylase, whereas PBS was used as a control. The dose of amylolytic activity used was typical of the minimal activity in the intestine (35 units/mL) (*34*). Fifteen milligrams of the complex was incubated in 1 mL of pancreatic amylase at 37 °C for 24 h. Pancreatic solution was prepared by dissolving 0.177 g of pancreatin in 20 mL of phosphate buffer (20 mM phosphate, pH 6.9, and 10 mM NaCl). PBS (159 mL) was added to the complex and centrifuged (5500*g*, 15 min), and the genistein was quantified by HPLC.

Release of Genistein. (A) Impact of Different pH Values and Temperature on the Release of Genistein. Complexes produced earlier were subjected to pH and temperature tests. Fifteen milligrams of the complex was incubated for 24 h in different pH values from 3 to 8, using phosphate buffer (0.2 M) or citrate buffer (0.1 M) at 30 °C. For the temperature stability studies, the complexes were incubated at three temperatures: 30, 50, and 80 °C for 24 h. The samples were diluted with PBS (159 mL), and the amount of genistein released from the complex was determined by HPLC as mentioned before.

(B) Stability in Simulated Stomach Conditions. The release of genistein from the complex was tested by incubating amylose or HACS complexes with genistein in simulated stomach conditions and following the amounts of genistein being released. The complex (15 mg) was incubated with 1 mL of HCl, pH 2, for 2 h at 37 °C under moderate stirring (15 rpm). The samples were diluted with PBS (159 mL), and the extent of genistein release was measured by HPLC as mentioned before.

(C) Enzymatic Digestion. The purpose of the test was to quantify the amount of genistein released from the complexes by enzymatic digestion as in the intestine. Fifteen milligrams of the complex was incubated in 1 mL of pancreatic amylase (35 units/mL) at 37 °C for 24 h as mentioned before. The amount of genistein released from the complex was calculated on the basis of the total genistein content in the complex. The amount of genistein released was compared to the amount that was released in PBS solution without the amylase (24 h at 37 °C).

Data and Statistical Analysis. All experiments were performed with at least three true repetitions, and results hereto are expressed as their means ± the standard deviation (SD). When necessary, the number of repetitions is noted in the text. The significance of the differences between groups was tested using *t*-test analysis. A probability level (p value) of <0.05 was considered to be statistically significant unless stated otherwise. Statistical analysis was performed by the data analysis tool pack of Microsoft Excel 2003 software.

RESULTS AND DISSCUSSION

X-ray Diffraction. To investigate the complex structure, the crystalline nature of the complex was determined by the position of the X-ray diffraction peaks. In this research, amylose, processed in a similar way but without a guest molecule, was used as a reference. XRD examination of the complexes, with genistein as guest molecule and amylose as a host, showed a diffraction pattern different from the one obtained from the reference (**Figure 2d**), pure genistein (**Figure 2a**), and from that of amylose−genistein physical mixture (**Figure 2e**). This diffractogram indicates the formation of a new structure in the presence of genistein.

The amylose genistein complexes showed reflections corresponding to the Bragg angles (2θ) at 7°, 8°, 10°, 14°, 15.5°, 17°, 23°, 25.5°, and 29° (**Figure 2b**). This pattern fits into the structure of $V6_{III}$ complexes suggested by Biais et al. and Buleon et al. (*7, 18*). In this structure there is a larger space between the helices, which indicates the location of the genistein as being between the helices. The $V6_{III}$ structure was suggested for a variety of guest molecules such as menthone and linalool (*17*). However, for small aromatic molecules, such as salicylic acid and its analogues, and molecules with two aromatic rings, such as naphthol or quinoline, a structure of seven or eight glucose residues per turn was suggested (*5, 9, 12, 14, 20, 21*). In these forms the guest molecule resides inside the helix structure. This study suggests that genistein, a bulky and aromatic molecule,

Table 2. Release of Genistein from the Complexes in PBS and by Enzymatic Digestion

	released genistein			
	PBS solution		pancreatic solution	
genistein−amylose ratio (w/w)	of total solid[a] (mg of genistein released/100 mg of complex)	of total genistein in complex[b] (%)	of total solid[a] (mg of genistein released/100 mg of complex)	of total genistein in complex[b] (%)
1/60	0.23 ± 0.03	15.8 ± 4.0	0.37 ± 0.03	26.2 ± 5.7
1/30	0.45 ± 0.02	22.4 ± 6.5	0.49 ± 0.05	24.8 ± 7.5
1/20	0.47 ± 0.11	15.7 ± 4.1	0.81 ± 0.21	27.1 ± 7.5
1/15	0.71 ± 0.31	13.6 ± 6.3	1.35 ± 0.33	25.8 ± 12.0
1/12	0.84 ± 0.23	7.8 ± 2.5	2.80 ± 0.52	25.9 ± 7.9
1/10	1.11 ± 0.33	9.9 ± 4.7	2.60 ± 0.30	23.0 ± 7.3

[a] Genistein release was calculated per 100 mg of complex. [b] Genistein release was calculated per total genistein content in the complex.

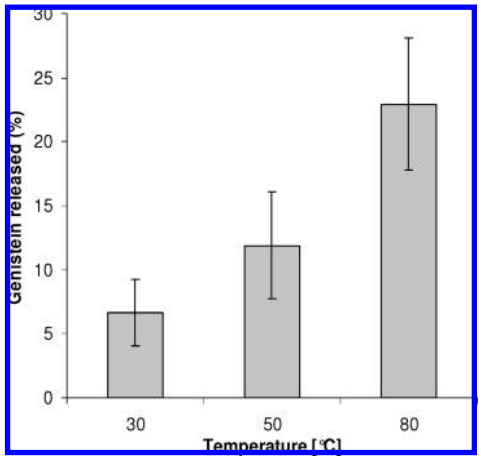

Figure 5. Release of genistein from amylose−genistein complexes at 30, 50, and 80 °C. Released genistein is expressed as percent of total genistein in the complex. The releases at the three temperatures are significantly different ($p < 0.05$).

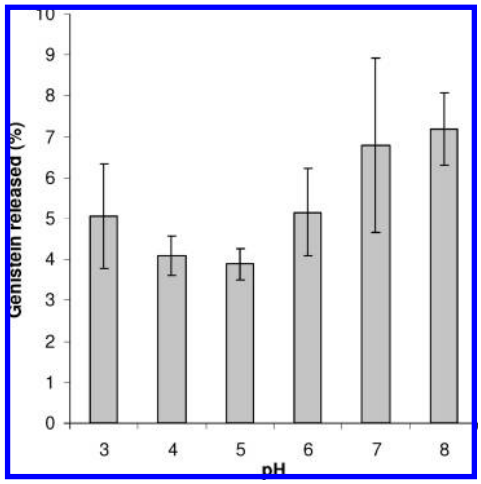

Figure 6. Release of genistein from amylose−genistein complexes under different pH values. Released genistein is expressed as percentage of total genistein in the complex. The release of genistein between pH 4−5 and 8 is significantly difference ($p < 0.05$).

induces the $V6_{III}$ structure, in which the guest is trapped physically between the amylose helices.

Because our efforts were focused on the possible use of these complexes as an encapsulation matrix in food applications, the use of starch instead of pure amylose was necessary. The procedure of self-assembly of amylose with guest molecule was formed to produce similar complexes with native starch (*3, 35, 36*). This use of starch could enable the use of techniques developed

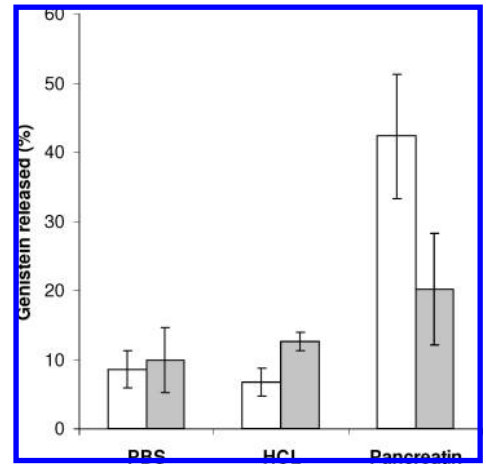

Figure 7. Release of genistein in simulated gastrointestinal conditions from amylose complexes (gray bars) or HACS complexes (white bars) in HCl solution (2 h) and by pancreatic amylase (24 h) as compared to spontaneous release in PBS (24 h). The amount of genistein released was calculated from the total genistein amount (percent). The release of genistein by pancreatic amylase is significantly higher than in HCl and PBS solutions ($p < 0.01$).

by our group for this purpose (*35, 36*). XRD diffraction patterns characteristic of HACS−genistein complexes and HACS without guest are shown in **Figure 2c,f**, respectively. HACS−genistein complexes also resulted in a $V6_{III}$ structure. This diffraction pattern is characterized by main peaks at Bragg angles (2θ) of 7°, 8°, 10°, 14°, 15.5°, 17°, 20°, 25.5°, and 29°. The additional peak at $2\theta = 20°$ compared to amylose−genistein diffraction patterns is similar to that found in HACS with no guest. This peak may correspond to internal lipid residues or petroleum ether residues in the HACS, which complexed some part of the amylose (*13, 32*).

Particle Size Analysis. Dynamic light scattering (DLS) and a laser scattering based particle sizing (Coulter Counter) were used to determine the particle size distribution. The size distribution was analyzed for complexes with pure amylose and complexes with HACS. Coulter Counter particle size analysis of amylose complexes showed particles of about 93 ± 36 μm. Amylose particles with no guest were too small to analyze using the Coulter Counter. Therefore, the use of DLS was necessary. The amylose particle size, without the guest molecule, was 148 ± 101 nm, about 2 orders smaller than with genistein. One may suggest that in an acidic environment, the presence of genistein induces hydrophobic interactions and that the resulting complexes tend to aggregate. This, however, was not supported by particle size analyses following an ultrasonic treatment (results not shown).

HACS particles without guest had a larger average diameter than amylose particles, possibly because some of the starch granules were still intact in the solution. HACS without guest resulted in a particle size of 24.24 ± 9.32 μm, similar to HACS particles with genistein (23.09 ± 11.46 μm).

Genistein Content. The total amount of genistein in the complex was measured by total dissolution of the complexes in KOH solution and quantified using HPLC. The total amount of genistein in HACS complexes was significantly smaller than the total genistein amount in the amylose complexes ($p < 0.01$). Eleven milligrams of genistein (11.3 ± 1.7 mg) was obtained from 100 mg of complex with amylose ($n = 22$), whereas HACS complexes contained 9.3 ± 1.5 mg of genistein/100 mg of complex ($n = 16$). Whereas both amylose and amylopectin substances can form inclusion complexes with the guest molecules, their organization differs (*4, 6*). HACS contains about 70% amylose and about 30% amylopectin. Because only long branches in amylopectin are able to form helices, their ability to form complexes is limited. HACS may also contain lipid residues, which remain after the defating process, or solvent remnants, which may occupy part of the amylose chain (*32*).

Effect of Different Genistein−Amylose Ratios. Better understanding of genistein−amylose conformation was pursued by preparing and characterizing complexes with different ratios of genistein−amylose (1/60, 1/30, 1/20, 1/15, 1/12, and 1/10). The influence of increased ratio of genistein−amylose on complex structure was studied by X-ray diffraction (**Figure 3**). A genistein−amylose ratio of 1/60 (**Figure 3a**) resulted in a B form, similar to amylose without a guest molecule (**Figure 2d**). Apparently, the genistein amount was too low to compete with amylose−amylose interactions, which form the double helices required for B form formation. Increasing the genistein−amylose ratio above 1/60 induced a $V6_{III}$ amylose structure. This indeed supports the suggestion that the formations of V- and B-forms compete; therefore, a mixture of single and double helices is formed, depending on the amount of genistein. Lebail et al. (*37*) showed that such competition over amylose fatty acid complexes reduces the overall crystalline order in the sample.

Along with the increase of the genistein−amylose ratio in the complexation solution, genistein content in the complex increased, ranging from 1.4% in 1/60 genistein−amylose ratio, and to 11.3% in 1/10 ratio (**Table 1**). Glucose−genistein molar ratio in the complexes indicates that in order to form the complex, 1 genistein molecule requires a minimum of 13 glucose molecules. This ratio fits well in the range of 8−25.2 glucose molecules per guest molecule, which is reported in the literature for other compounds and amylose (*1, 34*).

Complexes of different genistein−amylose ratios were also examined for their particle size distribution. At 1/60 ratio, the particle size was similar to amylose with no guest (about 127 nm), corroborating the observations made by the X-ray results (**Figure 3a**). At 1/15 to 1/10 ratios, the particle diameter was larger, about 81 μm, suggesting that the addition of genistein induces hydrophobic interactions between particles. At ratios of 1/30 and 1/20 the particles could not be detected properly by DLS (due to the high turbidity) or by the Coulter Counter (due to their low concentration). **Figure 4** demonstrates that as the genistein−amylose ratio increases, the turbidity caused by the complexes increases. From the particle size analysis (**Table 1**) and the image of the complexation solutions (**Figure 4**) one can suggest that increasing amounts of genistein induce the formation of larger particles.

Examining the pattern of genistein release can provide an interesting observation on the mode of its entrapment in the complex. Incubating the complexes in PBS solution resulted in low release of genistein (**Table 2**). As the genistein−amylose ratio increased, enzymatic digestion of the complexes released higher amounts of genistein ($r^2 = 0.9$, $p < 0.01$). However, the percent of genistein, being released out of the total genistein in the complex, did not change along with the overall genistein content. Therefore, it is possible that genistein is entrapped in the complex in three modes: weak (released spontaneously in PBS), moderate (released by mild enzymatic digestion), and strong (released by alkali dissolution).

On the basis of these results, the following model is hypothesized: the complexes contain two forms of helices, either B or V amylose. The ratio between the B form and the V form is determined by the amount of genistein added to the complexation solution. At a small genistein−amylose ratio the complexes contain mostly the B form. As this ratio increase, the two forms of the helix rival until the V form dominates. In the V form, genistein can be arranged in three different ways: in the amorphous regions, between the crystalline segments, or inside the crystals. In the amorphous parts, the molecules are released spontaneously in PBS. Mild enzymatic digestion likely releases the genistein trapped between the helices, whereas the genistein molecules that are securely trapped in the crystalline parts are released only by total dissolution of the complexes in KOH solution. Further studies are required to examine the validity of this hypothesis.

Thermal and pH Stability. Protecting the guest molecule from heat and low pH in the food matrix and stomach is imperative for the stability of a delivery system. The effect of temperature on the release of genistein was evaluated in PBS (pH 6.9) at 30, 50, and 80 °C. The release of genistein was calculated as a percentage from the total amount of genistein released in KOH solution. As shown in **Figure 5** the spontaneous release of genistein rises with temperature, starting from <7% at 30 °C to about 25% at 80 °C ($n = 3$). In addition, the complexes were stable under different pH values, with <10% of the complexed genistein being released. Between pH 4−5 and 8 significant differences in the amount of genistein released were observed (**Figure 6**) ($n = 3$, $p < 0.05$). The relatively higher percentage of genistein release at pH values as high as 8 can be explained by the alkali conditions, which may result in partial complex dissolution. At these pH values, the amylose helices and genistein molecules are partially charged; therefore, the hydrostatic interactions with the water increase and the interactions between amylose helices and genistein molecules decrease. As a result, the content of free genistein in the solution increases.

Stability in Simulated Stomach Conditions. One of the reasons for genistein complexation is to prevent its early release in the gastrointestine. Therefore, the stability of the complex was measured in simulated stomach conditions (HCl, 1 M, 2 h, 37 °C). For both HACS and amylose complexes, the release of genistein in acidic conditions was <15% of the total amount of genistein in the complexes (**Figure 7**) ($n = 5$). Using similar complexation process, Lalush et al. (*3*) showed that complexes of amylose with conjugated linoleic acid (CLA) were stable in the stomach. The CLA complexes, produced at 30 °C, showed <6% release of CLA from the total CLA. The method of complexation indicates that both amylose and HACS protect genistein from being released in simulated stomach conditions. Kim et al. (*38*) tested the effect of simulated stomach conditions on the isoflavones released

from microcapsules based on medium-chain triacylglycerol (MCT). In their test at pH values 2−5 the capsule showed between 12.5 and 15.8% genistein release after 1 h of incubation. By comparison to the present results, it can be seen that in the amylose-based complexes the release rate in simulated stomach conditions was even lower.

Enzymatic Digestion. The ultimate goal of this delivery system is to deliver nutrients to specific sites in the digestive tract, to protect them in the acidic environment of the stomach, and eventually to release them in the gastrointestine due to enzymatic hydrolysis of the amylose. The complexes were incubated in pancreatic solution, which contained the amylose-degrading enzymes, to quantify the amount of genistein released from the complexes. The amount of genistein released was compared to the amount released spontaneously in PBS (24 h at 37 °C). In the HACS complexes, 42.3 ± 9.0% of genistein was released by enzymatic digestion, whereas in PBS only 8.6 ± 2.7% of genistein was released (**Figure 7**) ($n = 5$). In the amylose complexes, 23.0 ± 7.3% of total genistein was released by enzymatic digestion, whereas in PBS only 9.9 ± 4.7% of genistein was released (**Figure 7**) ($n = 5$). These results demonstrate that although the genistein molecules are not located inside the helices, they are tightly physically entrapped between them. This location provides the genistein molecules protection from the acidic environment of the stomach while ensuring greater release under enzymatic digestion.

The spontaneous release of genistein, from HACS complexes and amylose complexes, showed no significant difference, which may indicate that genistein in the two complexes was trapped in a similar manner in the amorphous regions. However, the percentage of genistein release from HACS complexes after enzymatic digestion was significantly higher than in amylose complexes ($p < 0.01$). The faster release of genistein from the HACS particles may be explained by the observation that amylose−genistein particles were larger than HACS−genistein particles. The larger the size of the particles, the smaller the surface area to volume ratio, and therefore the potential surface to be hydrolyzed by enzymes decreases (*39*).

Therefore, HACS−genistein particles produced by complexation can be proposed as a slow-release system. The efficacy of this delivery system should yet be tested in vivo.

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