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Detection of a Novel Three Component Complex Consisting of Starch, Protein, and Free Fatty Acids

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A water soluble three way complex composed of starch, whey protein, and free fatty acid (FFA) was detected in a dilute three component system after heating. In high-performance size exclusion chromatography (HPSEC) profiles of the starch−protein−FFA system, the three way complex eluted between amylopectin and amylose. The molecular mass of the complex, based on multiangle laser light scattering/HPSEC and pullulan standards, was estimated to be ∼(6−7) × 10^6 Da. Carbohydrate measurement by the phenol−sulfuric acid method clearly showed that the starch amylose fraction shifted to a higher molecular weight elution volume following complexation. Whey protein existed as large disulfide-linked aggregates and is speculated to be the organizer of the three way complex. Differential scanning calorimetry of the freeze-dried complex showed the presence of an amylose−FFA melting endotherm, thus proving that FFA was the third component in the three way complex and that the amylose−FFA complex was one of the structural components of the complex. The complexation mechanism and its relationship with changes in starch functionality were discussed.

KEYWORDS: Starch; protein; free fatty acid; interaction; complexation

INTRODUCTION

Starch, protein, and lipids are three major components of many food systems. Interaction among these food components plays an important role in food quality, particularly on the texture and mechanical properties of food products. The existence of a three way interaction among starch, soluble protein, and free fatty acid (FFA) and its affect on functionality were recently established in our laboratory (*1*). Using a Rapid Viscoanalyzer (RVA), a high paste viscosity cooling stage peak was produced when whey protein and FFA were combined with starch. Characteristics of the cooling stage viscosity peak were affected by the relative quantity of each component, the sequence of addition of each component into the system, and the molecular structure of the FFAs. Moreover, amylose was found to be involved, as waxy starch did not produce the RVA cooling stage peak. In other work in our laboratory, whey protein was shown to compete with amylose for the binding of FFAs as demonstrated through reduction of the amylose−FFA melting endotherm when the protein was present (Zhang and Hamaker, unpublished data). This follows numerous reports of FFA binding to β-lactoglobulin (*2*, *3*) and indicates that the two component complexes, starch−FFA and protein−FFA, are common in the three component system.

It is well-known that functions of food polymers are related to their structures. The key to understanding the formation of the RVA cooling stage viscosity peak was to find the structure formed during the three way interaction. Starch, protein, and FFA are the three primary structural elements of the three component system, and it was established in related work that starch−FFA and protein−FFA complexes are secondary structural elements formed during the three way interaction. On the basis of these findings, the possible formation of a new ternary structure was investigated in a dilute three component system after cooking using high performance size exclusion chromatography (HPSEC).

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MATERIALS AND METHODS

A normal sorghum cultivar, P721N, was harvested from the Purdue University Agronomy Farm in 1997 and was conditioned (27 °C, 67% relative humidity) for 2 weeks to approximately 13% moisture content. Starch was isolated from whole sorghum grain according to a general toluene procedure for starch isolation (*4*). Starch was defatted with 85% methanol for 16 h at room temperature. Whey protein isolate (WPI-BioPro, 97.5% protein) was from Davisco Foods International, Inc. (Eden Prairie, MN). Palmitic (C16:0), oleic (*cis*-9-octadecenoic acid, C18:1), and linoleic (*cis*-9,*cis*-12-octadecenoic acid, C18:2) acids were from Sigma Chemical Co. (St. Louis, MO). Purified water was obtained from a Barnstead 3 module E-pure, organic free system (Dubuque, IA).

Starch, Protein, and FFA Complexation in a Dilute System. A dilute system was used to examine starch−protein−FFA complexation. Starch concentration was 0.5% (w/v), whey protein concentration was 10% (w/w, based on starch), and FFA concentration was 5% (w/w, based on starch). The starch solution (0.5%) was prepared by heating for 20 min in a boiling water bath and was then cooled overnight at room temperature. After it was centrifuged at 14000*g* for 20 min, the supernatant was further purified by ultracentrifugation at 100000*g* for 1 h at 20 °C. The supernatant was then used as the starch sample for

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interacting with protein and/or FFA. Interaction among starch, FFA, and protein was achieved by adding 5 mg of whey protein (dissolved in purified water, 10 mg/mL) and/or 2.5 mg of FFA (palmitic, oleic, and linoleic acids, dissolved in petroleum ether, 10 mg/mL) to a 10 mL starch solution sample and then cooked for another 20 min in a boiling water bath. The protein control was similarly cooked for 20 min. After the samples were cooled overnight at room temperature, the supernatant following centrifugation at 14000*g* for 20 min was filtered through a 0.45 μm filter and injected into a HPSEC system (described below) for analysis.

Differential Scanning Calorimetry (DSC). A differential scanning calorimeter, DSC 2920 (TA Instruments, New Castle, DE), was used to examine starch−lipid complexation. The complexed samples (supernatant from final step of above procedure) were frozen in liquid nitrogen and were then freeze-dried. Yield represented the ratio of the amount of dried material to the amount of the starch (dry basis) added before preparing the samples. The starch content was measured by the phenol sulfuric acid method (*5*) after dissolving the freeze-dried samples in purified water (1 mg/mL) and expressed in the table as relative content based on the starch control samples (as 100%). Dried samples (3−5 mg) and purified water (1:3, w/w) were hermetically sealed in aluminum pans. Experiments were carried out at a heating rate of 10 °C/min from 20 to 130 °C and then cooled to 20 °C at the same rate. The value of enthalpy (J/g starch) is the value of measured enthalpy divided by starch content (dry weight basis).

HPSEC. The chromatography system consisted of a Varian model 9012 HPLC pump (Walnut Creek, CA), a HR 16/50 (Pharmacia, Sweden) column packed with Sephacryl S 500 HR (Pharmacia), a UV−vis detector (Varian model 9050), and a refractive index detector (Varian model 9040). The mobile phase was filtered (0.22 μm filter) and degassed with purified water containing 0.02% sodium azide. The eluent at a flow rate of 1.5 mL/min first passed through the UV−vis detector at 280 nm and then the RI detector. A 1 mL sample loop was used. Column backpressure was 6−8 atm. Flow cell temperature of the refractive index detector was maintained at 30 °C. Data from the refractive index and UV−vis detectors were collected and analyzed using the Varian Star 4.5 software program. The eluent was collected at 2 min/tube. The phenol−sulfuric acid method (*5*) was used to measure total carbohydrate content, and the blue value was measured at 620 nm after mixing 0.2 mL of iodine solution (0.2% I_2, 2% KI) with 2.5 mL of eluent. Pullulan standards (Polymer Laboratories, Inc., Amherst, MA; molecular masses (Da): 788 000, 404 000, 212 000, 112 000, 47 300, and 22 800) were used to obtain a general estimate of molecular weight of the three component complex. A multiangle laser light scattering detector (MALLS, Dawn DSP-F, Wyatt Technology, Santa Barbara, CA) was used to determine absolute molecular mass of the complex. The MALLS detector had a K5-cell and a wavelength of 488 nm. The calibration constant for the detector (1.151E-04) was obtained by measuring the light scattering intensity of toluene at a scattering angle of 90° (detector 11) and normalizing with bovine serum albumin.

RESULTS AND DISCUSSION

HPSEC Profiles of One and Two Component Systems. A representative HPSEC RI profile of water soluble cooked starch had one sharp amylopectin peak eluting at the column void volume (at about 30 min) followed by a broad amylose peak from 40 to 60 min (**Figure 1A**). There was some absorbency at 280 nm in the amylopectin region of the chromatogram, presumed to be due to slight turbidity caused by the eluting amylopectin fraction. The HPSEC profile of cooked whey protein showed a small peak at the void volume and one significant peak at about 58 min using both UV and RI detection. It has been shown that whey protein, of which the major β-lactoglobulin component has a molecular mass of approximately 34 kDa, forms large aggregates through disulfide bond (S−S) linkages upon cooking (*6*). β-Lactoglobulin forms aggregates up to 4×10^6 Da (*7*). While 2-mercaptoethanol (ME), a strong reducing agent, cleaves intermolecular S−S linkages to reduce whey protein aggregates to their monomers, at the same time, denatured whey protein can form another type of aggregate through hydrophobic interaction. This may have been the reason that two peaks appeared in the UV signal profile after treatment with 1% ME, one at the void volume and the other at 70 min. In the RI profile, there was only one small peak observed at the void volume (**Figure 1B**), although a

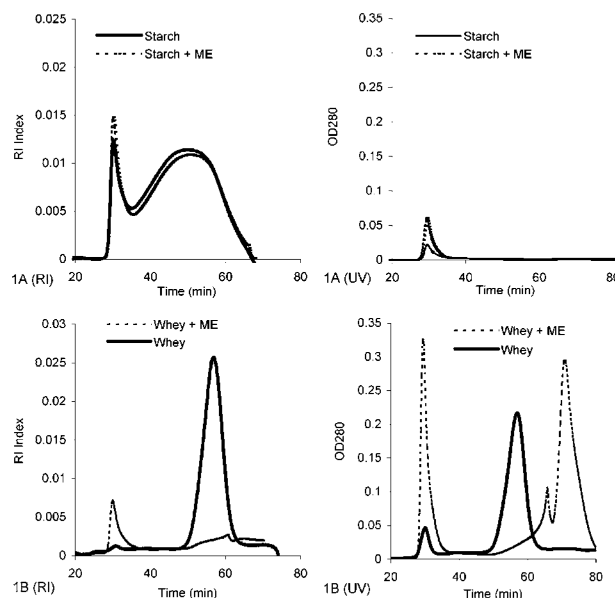

Figure 1. HPSEC profiles from RI and UV signals of starch (**A**) and whey protein (**B**). Concentrations are the same as in other samples.

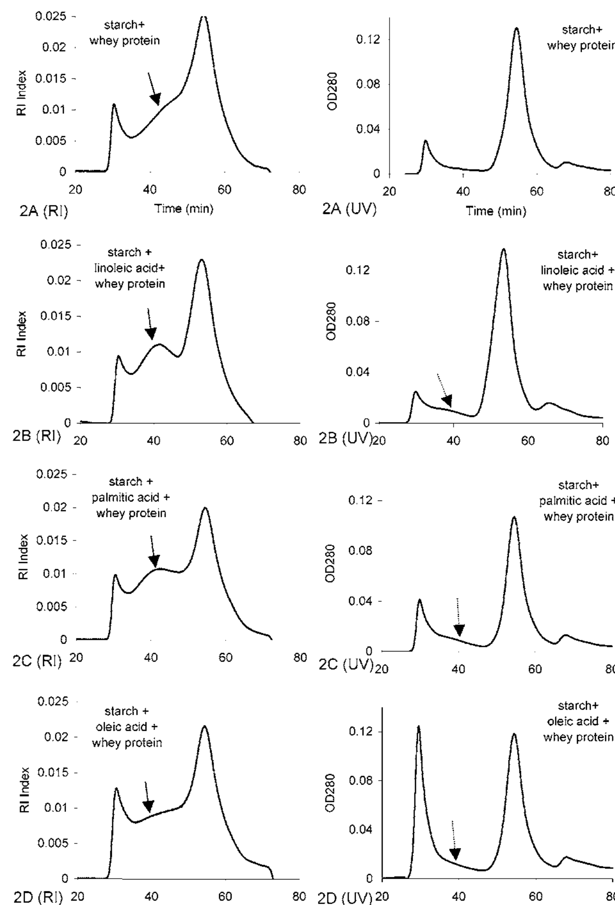

Figure 2. HPSEC profiles from RI and UV signals of starch/whey samples without and with FFAs.

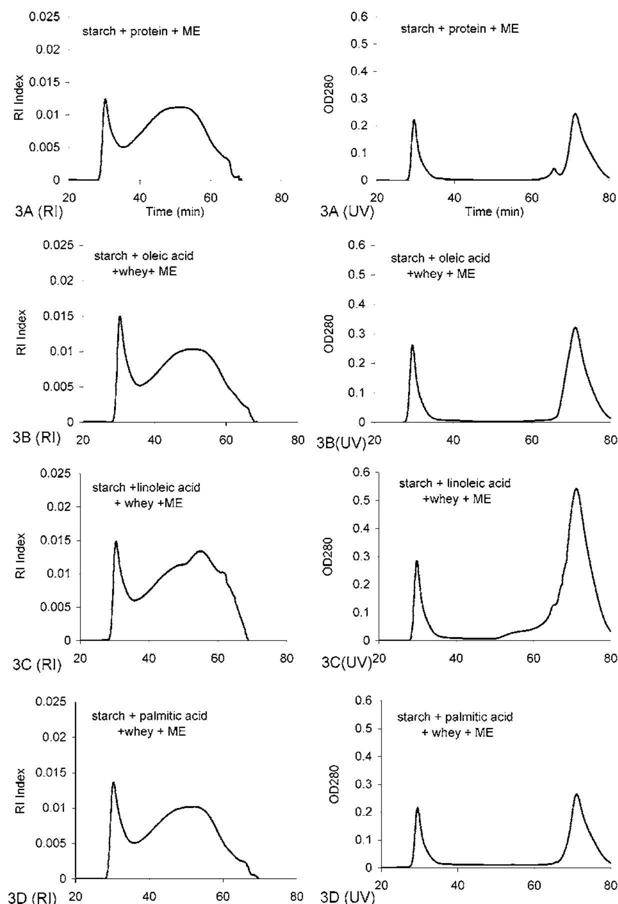

Figure 3. HPSEC profiles from RI and UV signals of starch/whey samples without and with FFAs after treatment with 1% ME.

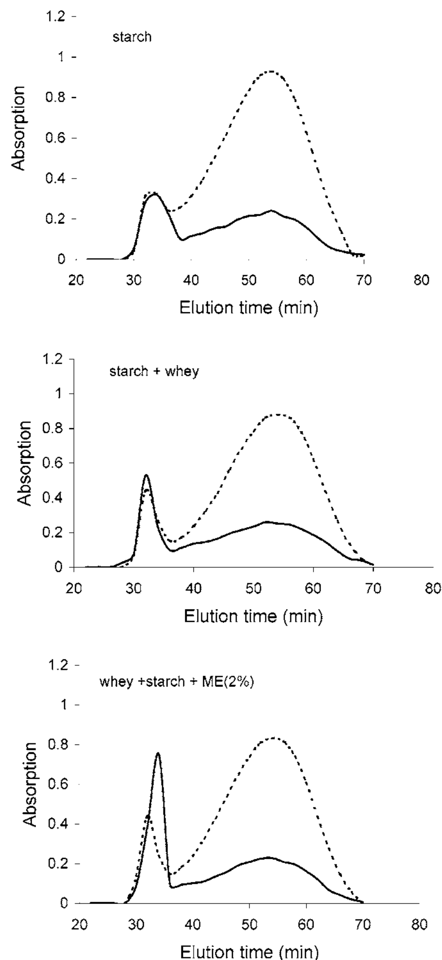

Figure 4. HPSEC profiles of starch and starch with whey protein. The dotted line denotes OD 620 nm (blue value). The solid line denotes total CHO content (OD 490 nm).

negative RI peak at around 70 min elution time may have masked a second peak. For water soluble cooked starch, addition of 1% ME slightly increased the height of the first amylopectin peak in the UV profile without affecting the amylose peak; there was no effect on the RI profile (**Figure 1A**). It was important to the study that ME removed the whey protein aggregates that appeared around the 58 min elution volume and, thereby, did not interfere with the amylose peak in RI profiles in subsequent experiments where ME was used.

The addition of whey protein to starch produced a large protein peak at about 58 min elution time as seen in both the RI and the UV profiles (**Figure 2A**) that coincided with that observed in **Figure 1B**. The high molecular weight (HMW) amylose portion of the peak was still visible (**Figure 2A**, see arrow). After treatment with ME, the protein signals separated completely from the amylose peak and the RI elution profile of starch + whey protein (**Figure 3A**) appeared similar to starch alone (**Figure 1A**). Thus, the reducing agent did not affect the carbohydrate portion of the HPSEC profile of the starch + whey sample. Neither did whey protein change the HPSEC profile of soluble cooked starch molecules measured by the phenol−sulfuric acid method (**Figure 4**). These experiments showed no observable interaction between starch and whey protein.

When starch was cooked with FFA, only the amylopectin peak appeared in the elution profiles with little or no second eluting amylose peak (**Figure 5**). This was caused by precipitation of amylose−FFA complexes, because the amylose−FFA complex, like most starch−lipid complexes, is water insoluble at pH $\leq$ 7 (*8*). Amylose−FFA complexation occurred in the dilute system used in this study, suggesting that interaction between amylose and FFA is an important factor when considering the three way interaction among starch, protein, and FFA.

HPSEC Profiles of Three Component Systems and Complexation. Prepared starch samples cooked in the presence of whey protein and FFA produced supernatant HPSEC elution profiles substantially different as compared to profiles of one or two component samples (**Figure 2**). A new molecular complex was found in RI profiles that eluted at the HMW end of the amylose profile (**Figure 2**, see arrows in three component profiles). The molecular mass of the new complex was (6−7) $\times$ 10^6 Da, as estimated by MALLS/HPSEC (**Figure 6**). This molecular mass range was supported by pullulan standards (not shown). Comparison of RI profiles between the starch + protein (**Figure 2A**) and the starch + FFA + protein (**Figure 2B−D**) shows that all three components were required to produce the complex. No indication of the complex was observed in the starch + protein profiles (**Figure 2A**). Linoleic acid produced the highest amount of complex, followed by palmitic and oleic acids. While RI profiles showed the complex most clearly, the presence of the complex could also be seen in the UV profiles, thus verifying that protein was part of the complex (**Figure 2B−D**, see curved arrows). Starch, protein, and FFA were clearly the structural elements of the novel ternary complex.

Whey protein aggregates formed through disulfide bond linkages appeared to be the main structural organizer of the new complex. After treatment with 1% ME, complex detection in HPSEC RI and UV elution profiles disappeared (**Figure 3B−

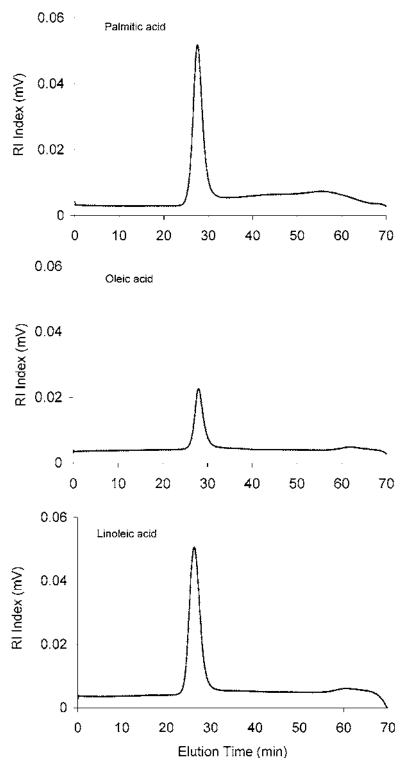

Figure 5. HPSEC profiles from RI signals of starch in the presence of different FFAs.

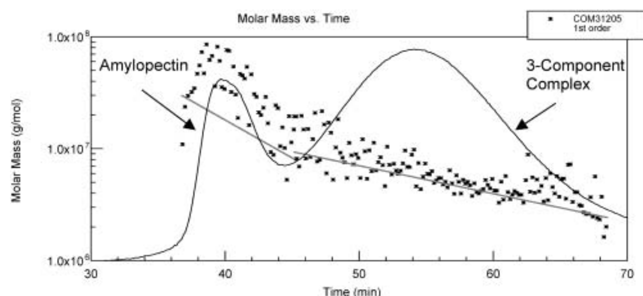

Figure 6. Molar mass vs elution time of amylopectin and the three component complex determined using the MALLS detector (dots are molar mass, solid line is RI profile). Note that molar mass of amylopectin was between 10^7 and 10^8 Da. The molar mass of the three component complex was ~(6–7) × 10^6 Da; flow rate, 1 mL/min.

D), resulting in profiles very similar to those of control samples (starch, starch + whey protein). Therefore, reduction of disulfide-bound polymers to protein monomers caused disruption of the complex.

HPSEC elution profiles of the three component system before and after treatment with ME as measured by phenol−sulfuric acid and the blue value provided supporting evidence for the existence of the three way complex (**Figure 7**) and also showed that amylose was the participating starch molecule in the three way complex. In these experiments, HMW starch peaks representing the complex at the trailing edge of the amylopectin void volume peak were separated by 1% ME treatment into two distinct peaks typical of amylopectin and amylose (**Figure 7**), while no changes were found in the elution profiles for the starch + whey sample (**Figure 4**) before and after ME treatment. ME had no effect on starch alone (**Figure 1**). After ME treatment, the newly appearing second peak was amylose as shown by its elution profile and its high blue values (**Figure 7**). Its increase in molecular mass in the three component system was due to complexation with the other two components. In

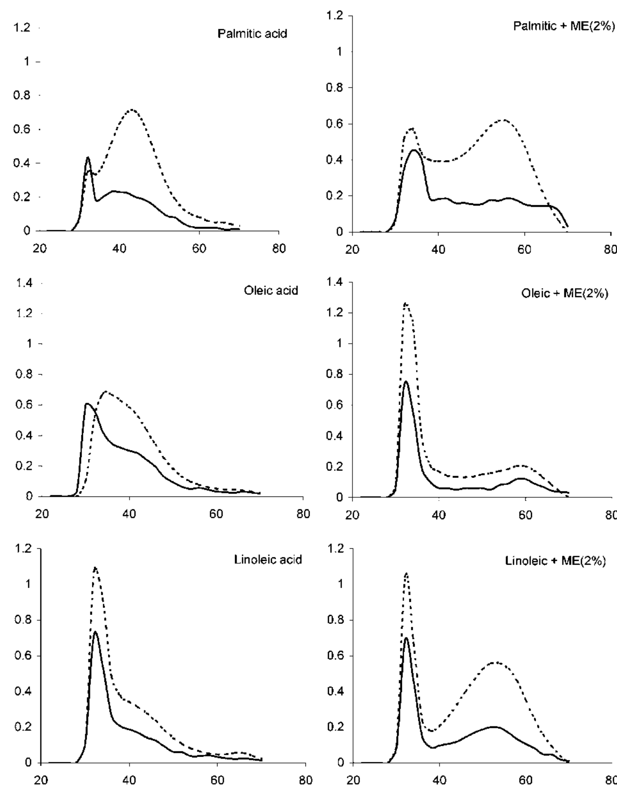

Figure 7. HPSEC profiles of starch samples in the presence of whey protein and different FFAs. The *X*-axis is the elution time (from 20 min). The *Y*-axis is the OD value at 620 (dotted line) and 490 nm (solid line).

summary, the above data show clearly that a water soluble three component complex was formed through interaction among starch (amylose), soluble protein, and FFA.

Starch−FFA Complexes in the Three Component Complex. As noted above, the formation of this novel three way complex was not through specific binding between starch molecules and whey proteins. Obviously, FFA molecules played a critical role in the three way complex formation. In this regard, it is well-known that whey protein binds FFA molecules (*2*, *3*) and that starch−FFA inclusion complexes form between amylose and FFAs. Amylose was shown above to be part of the complex, and in a previous paper (*1*), we reported only nonwaxy starches resulted in the three component interaction. However, further evidence for amylose−FFA complexation within the three way complex was needed, due to the fact that amylose−FFA complexes are normally water insoluble, while the three way complex was water soluble. DSC was used to study the contribution of the amylose−FFA complex in the formation of the three way complex.

DSC data of freeze-dried water soluble materials after the three way complexation showed that FFA was complexed with amylose (**Table 1**). Samples that contained starch, whey protein, and FFA produced a large endothermic peak at ~100 °C upon heating (due to melting of the amylose−FFA complex) and a large exothermic peak at ~75 °C during cooling (due to amylose−FFA complex reformation). These two peaks are characteristic of loss and formation of starch−lipid complexes. Isolated starch itself showed low enthalpic transitions that were due to native amylose−lipid complexes, and addition of whey protein to starch resulted in a reduction of the complex presumably due to competitive lipid binding by the protein.

Yield of the three component complex (**Table 1**) represents the ratio of the amount of dried material to the amount of the starch added before preparing the samples. The values obtained

Table 1. Starch Content, Yield, and DSC Results of Water Soluble Materials from the Three Way Interaction

samples	starch (S) content (%)	yield (%)	process (H/C[a])	temp (°C) onset	temp (°C) peak	enthalpy (Δ*H*) (J/g S)
	H	92.4	102.6	2.34		
S control	100.0	27.5	C	83.5	79.4	2.20
	H	94.1	101.7	0.72		
S + whey (W)	85.0	29.1	C	79.5	76.2	0.80
	H	96.3	103.1	8.87		
S + W+ palmitic	75.8	32.6	C	82.1	79.2	8.61
	H	92.1	101.0	8.28		
S + W + oleic	83.0	35.2	C	81.2	78.0	6.69
	H	90.3	97.2	8.21		
S + W + linoleic	80.7	35.6	C	77.9	74.9	6.78

[a] H, heating; C, cooling.

by multiplying the yield by starch content for each sample represented the total amount of water soluble starch. These values were not notably different from each other. This indicates that most of the original water soluble starch component (primarily amylose) remained water soluble after complexation. Therefore, amylose−FFA complexes in a three component system were water soluble and most of the starch−FFA complex stayed in solution. Interaction with protein apparently provided the structure and increased water interaction to bring the starch−FFA complex into solution. It was observed that solubilities of the freeze-dried materials were different between control samples (starch, starch + whey protein) and three component samples. Freeze-dried materials from control samples required 90% dimethyl sulfoxide for dissolution, while materials from the three component system were easily dissolved in water. This provides other indirect evidence that indeed a three way complex formed.

Relationship between the Three Way Complex and the Three Way Interaction. In a previous paper (*1*), we showed that interaction among starch, protein, and FFA causes a high viscosity cooling stage peak on a RVA pasting profile. This relates the formation of the HMW three component complex reported here with a functional property change. The high water solubility of the three way complex means that it has an intimate interaction with water molecules in the system, thereby suggesting a large hydrodynamic volume of the starch−protein−FFA complex. We speculate that this large hydrated complex caused the noted increase the viscosity. Starch−FFA complexation occurs around ∼75 °C, as demonstrated by DSC during cooling. This was also the approximate temperature of the three way complex formation, as RVA paste viscosity began to increase at about this temperature followed by its characteristic peak. Although the amylose−FFA complex itself is known to increase cooling stage paste viscosity (or setback), presumably due to increase in radius of gyration of the amylose, there was no peak observed without protein, and viscosity increase was small as compared to addition to starch of oleic and linoleic acids with protein (palmitic acid with protein produced a lower viscosity cooling stage peak). Thus, the rapid increase in system viscosity appeared to start at the point where the starch−protein−FFA complex started to form. It was unclear why paste viscosity then peaked and decreased.

CONCLUSION

A novel ternary complex was found from three way interaction among starch, whey protein, and FFAs. The complex was identified by HPSEC with detection by refractive index and UV detectors and by carbohydrate and iodine-binding analyses. The structural basis of a three way interaction among starch, protein, and FFAs was established. Starch−FFA complexes were one structural element of the three way complex, presumably protein−FFA complexes another, and a disulfide bond-linked protein aggregate was an important structural element and the organizer of the three way complex. This three way complex combined starch and protein molecules, two important biopolymers that are inherently difficult to interact with each other. FFAs played an important role to bridge starch and protein, although specifically how this occurs is unclear. The three way complex was related as the structural basis for changes in starch pasting properties during the RVA cooling stage.

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