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ARTICLE

Free Fatty Acids Electronically Bridge the Self-Assembly of a Three-Component Nanocomplex Consisting of Amylose, Protein, and Free Fatty Acids

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The self-assembly of a ternary complex, which is formed through heating and cooling of a mixture of amylose (1.0 mg/mL), whey protein isolate (50 μg/mL), and free fatty acids (FFAs, 250 μg/mL) was investigated. High-performance size-exclusion chromatography–multi-angle laser light scattering (HPSEC–MALLS) analysis showed that the complex is a water-soluble supramolecule ($M_w = 6-7 \times 10^6$), with a radius of gyration of 20–100 nm, indicating a nanoscale complex. Experimental results using 1-monostearyl-rac-glycerol (MSG) or cetyl alcohol that is similar to FFA in structure (except the headgroup) indicate that FFAs are the bridge between thermodynamically incompatible amylose and protein molecules and their functional carboxyl group is essential to the formation of the complex. Additionally, the effects of pH and salt treatments suggest that electrostatic interactions between negatively charged carboxyl groups of FFAs and polyionic protein are the foundation for the self-assembly of the complex. The fact that FFA is one important component in the self-assembled complex with an estimated molar ratio of 6:1:192 (amylose/protein/FFA, ~4–5% FFA) demonstrates that it might be used as a nanocarrier for the controlled release of lipophilic functional materials to maintain their stability, bioactivity, and more importantly water solubility.

KEYWORDS: Soft matter; starch–FFA–protein complex; nanocarrier; self-assembly

INTRODUCTION

Starch, protein, and lipids are three common biological materials that belong to the category of soft condensed matter, also called soft matter, a newly emerged physics term concerning polymers, liquid crystals, colloids, emulsions, and supramolecules. A unique feature of soft matter is that its physical behavior, which predominantly occurs at a room-temperature thermal energy scale, cannot be predicted or is difficult to be predicted directly from its atomic or molecular constituents because of their inherent nature of complexity and flexibility (*1*). One of the most important properties of soft matters is their ability to "self-assemble" into complex physical structures. Self-assembly or bottom-up assembly is a spontaneous and reversible process to organize molecular units into ordered structures through noncovalent interactions (*2*) including hydrogen bonds, ionic interactions, dipolar interactions, van der Waals forces, and hydrophobic interactions. Nano- and mesoscopic physical structural units are the basic building blocks in the self-assembly of soft matters that can be used to design thermodynamically stable supramolecular entities with desired properties and functions. The combination of soft matter science and nanotechnology is termed soft nanotechnology (*3*), concerning the manipulation of soft matters into nanocomplexes through their inherent self-assembly capability.

Proteins and carbohydrates are thermodynamically incompatible (*4*) soft matters in nature, and phase separation between them often occurs even in concentrated systems. Recently, a water-soluble ternary complex resulting from the self-assembly of amylose, protein, and free fatty acid (FFA) was discovered in our group through the observation of a rheological viscosity peak at the cooling stage of Rapid ViscoAnalyzer (RVA) analysis of the three-component system (*5*). Further studies using a size-exclusion chromatography and multi-angle laser light scattering system (*6*) revealed a distinct water-soluble ternary complex consisting of amylose, protein, and FFAs with a molecular weight of 10^6-10^7 Da. The concomitant formation of the ternary complex and changes of RVA viscosity during the cooling process demonstrated that the water-soluble ternary complex, as a new structural component, affects the rheological property of the system. Experimental results using several cereal flours, in which starch, protein, and lipids (addition of FFAs) are present, also showed the existence of a ternary complex (*7*), implying that this complex might be a universal mode of interaction among the soft matters of starch, protein, and FFA.

The ternary complex could potentially be used as a carrier of lipophilic functional molecules as lipophilic FFA, mainly present

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in the form of an amylose−FFA sub-complex (*8*), is one important structure component of the complex. Similar to the conventional amylose−FFA complex that can carry bioactive FFA such as conjugated linoleic acid (*9*), and protect it from oxidation, the ternary complex can also carry and maintain the structural integrity of the lipophilic FFA with its amylose−FFA sub-complex. Additionally, the water solubility of the three-component complex, which is different from the insoluble nature of the conventional amylose−lipid complex (*10*), provides wide possibilities for its use in functional food development or drug delivery, such as incorporating functional lipophilic materials in beverages or being used as a drug delivery method for dissolution of water-insoluble drugs. The water solubility of the self-assembled ternary complex suggests its unique niche in delivering valuable lipophilic bioactive compounds.

Although we have shown the existence of the ternary complex, how the lipophilic core of helical amylose, polyionic protein, and the amphiphilic FFAs contribute to the self-assembly of the complex is still elusive. In this study, further investigation, particularly focusing on the function of the carboxyl group of FFA, was conducted to better control the self-assembly of these materials and to provide further understanding regarding its structure, physicochemical properties, and potential applications.

MATERIALS AND METHODS

Materials. Normal maize starch was provided by Tate and Lyle (Decatur, IL) and defatted with 85% methanol for 16 h at room temperature before analysis. Whey protein isolate (WPIBioPro, 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, β-lactoglobulin, 1-monostearyl-rac-glycerol (MSG), cetyl alcohol (C16:0), and α-amylase (EC 3.2.1.1) type VI-B from porcine pancreas (19.6 units/mg) were from Sigma Chemical Co. (St. Louis, MO). Purified water was obtained from a Barnstead 3 Module E-pure, organic-free system (Dubuque, IA). The bicinchoninic acid (BCA) protein assay kit was from Pierce Biotechnology, Inc. (Rockford, IL).

Amylose, Protein, and FFA Complexation in a Dilute System. A dilute system was used to examine amylose−protein−FFA complexation. The normal corn starch solution (0.5%) was prepared by heating for 20 min in a boiling water bath and was then cooled until room temperature. After it was centrifuged at 30,000*g* for 20 min, the supernatant was then used as the amylose solution for interacting with protein and/or FFA. Interaction among amylose, FFA, and protein was performed by adding 0.5 mg of whey protein or its main component β-lactoglobulin (dissolved in purified water, 10 mg/mL) and 2.5 mg of FFA (palmitic, oleic, or linoleic acids, dissolved in petroleum ether, 10 mg/mL) to a 10 mL amylose solution in a capped glass tube and then heated for another 20 min in a boiling water bath with continuous stirring. The sample was cooled slowly overnight until room temperature with continuous stirring, and the supernatant was filtered through a 0.45 μm filter and injected into a HPSEC−MALLS system (*11*) for analysis. The same procedure was used to prepare the amylose−FFA complex without adding the protein component.

To examine the function of the carboxyl group of FFA in self-assembly, cetyl alcohol and MSG that are structurally similar to FFA, except the headgroups, were used in the three-component system and the formation of the ternary complex with amylose and whey protein was measured by chromatography (*6*) and a rheological method according to the procedure by Zhang and Hamaker (*5*).

Particle Size Measurement. The size distribution of the ternary complex was measured using a ZetaSizer (Malvern Instruments Ltd., Worcestershire, U.K.) following the manual instructions. After filtration with a 5 μm filter, the ternary complex solution was used directly to measure the size (diameter), expressed as *Z* average in nanometers.

Salt and pH Treatment and Self-Assembly of the Ternary Complex. Two experiments were carried out for different purposes. First, the effect of pH and salt on the formation of the ternary complex was examined by setting the pH or salt concentration before the process of heating and cooling, and then the procedure described above was followed to measure the formation of the ternary complex. Second, water solubility

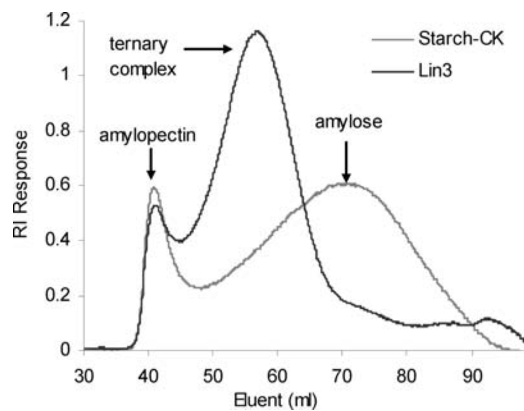

Figure 1. HPSEC profile of a typical ternary complex consisting of amylose, β-lactoglobulin, and linoleic acids. Starch-CK, starch control solution; Lin3, ternary complex when the FFA component is linoleic acid.

and the possible dissociation of the ternary complex as affected by pH and salt were examined after the complex was formed. The ternary complex collected from the column in the eluent (0.02% sodium azide, w/v) was concentrated by air blowing at room temperature until a precipitate appeared, and then it was dialyzed in purified water until the precipitate was solublized. The sodium chloride concentration (0.01−0.1%) or pH (2.5−8.0) of the concentrated complex solution was then altered accordingly. After centrifugation (10, 000 rpm, 2 min) using a microcentrifuge, OD_{620} representing iodine binding of the complex was measured on the basis of the procedure by Zhang and Hamaker (*8*) and the protein concentration in the supernatant was measured using the BCA kit according to the instructions. Experiments using RVA and differential scanning calorimetry (DSC) were carried out as described previously (*5*, *8*).

Digestion with Porcine Pancreatic α-Amylase. Susceptibility of the ternary complex to α-amylase digestion was measured by adding 4 mL of purified water, 1 mL of buffer of 10 mM sodium glycerophosphate (50 mM $CaCl_2$ at pH 6.9), and 0.05 unit/mL porcine pancreatic α-amylase to 5 mL of the complex solution and incubating at 37 °C. The samples (0.2 mL) obtained at different time intervals (0, 5, 10, 30, 60, 90, and 120 min) were placed in a microcentrifuge tube containing 20 μL of H_2SO_4 (2 M) to stop the enzyme reaction. The reducing sugar concentration (maltose equivalent) was measured according to the Nelson−Somogyi method (*12*). Digestibility of the samples was expressed as a percentage of the control with the highest degree of digestion.

RESULTS AND DISCUSSION

Conformation and Particle Size of the Ternary Complex. As previously shown (*6*), the major component of the initial starch solution after centrifugation was amylose with a broad distribution (starch-CK in **Figure 1**) and a small peak representing residual amylopectin. A ternary complex with a narrower size distribution ($M_w = 6-7 \times 10^6$ Da) was formed after a heating and cooling process when amylose, β-lactoglobulin (the major component of whey protein), and linoleic acid were present in the system (Lin3 in **Figure 1**). The disappearance of the amylose peak after the formation of the ternary complex demonstrates that amylose is the major component of the ternary complex. Similar HPSEC profiles were also shown when other FFAs, such as oleic, palmitic, or stearic acid, were used as the FFA component (data not shown). Therefore, the ternary complex formed among amylose, whey protein, and FFA was not unique to one specific FFA.

Although all of the FFAs used in experiments formed a ternary complex with the same amylose and protein, there was a high variation in their molecular weights (M_w) and radii of gyration (R_z) (data not shown). This variation not only occurred between different FFAs, but was also found within different batches using the same FFA component, which reflects the complexity and flexibility of the process that is characteristic of the self-assembly

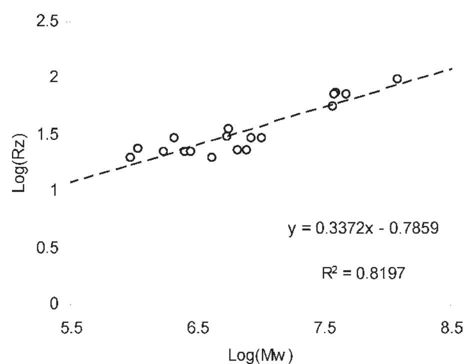

Figure 2. Relationship between the radius of gyration (R_z, in nanometers) and molecular weight (M_w) obtained from HPSEC−MALLS. The linear relationship with a slope of 0.337 indicates a spherical conformation of the ternary complex.

of soft matters (*13*). On the basis of these variations, a conformation plot was made using the logarithm of the MALLS-generated M_w and R_z (**Figure 2**). According to the theory of MALLS, a slope of 0.34 suggests that the complex approaches a spherical conformation in solution. As for the size of the complex, the R_z data showed that the complex is a nanoscale supramolecule. Consistently, the results of the laser-scattering measurement of the size of the complex, represented by the *Z*-average diameter, showed that a ternary complex with linoleic acid was ∼150 nm and a ternary complex with palmitic acid was ∼78 nm, while the control of the amylose and amylose/protein was 93 and 93 nm, respectively. The size difference of ternary complexes formed using linoleic and palmitic acids indicates the important role of FFA in the formation of the complex, and the difference in diameter with the control amylose suggests that the conformation of amylose in the complex has been changed because amylose in its dilute solution has a linear random-coil conformation (*14*). The finding that amylose alone and amylose + whey protein were of similar size supports the thermodynamic incompatibility between protein and amylose.

Estimation of the Molar Ratio of the Three Components in the Complex. Obtaining an estimation of the molar ratio of each component in the complex is essential to a better understanding of the structure of the complex and the function of each component in its formation. We have previously shown that the amylose−FFA complex is one important secondary structural component (*8*), and thus, the molar ratio between amylose and FFA could be estimated according the structure of the amylose−FFA complex, in which three amylose helical turns are required to complex with one fatty acid molecule and one helical turn has six glucosyl units (*15*). The average molecular weight (M_w) of amylose (**Figure 1**) based on the MALLS analysis is 200,000 Da (i.e., around 1230 glucosyl units in an amylose molecule). Therefore, the number of FFA molecules to saturate an amylose molecule, in theory, would be ∼68. Experimentally, Karkalas and Raphaelides (*16*) showed that, for stearic acid (18:0), about 9.5 mg of FFA is required to saturate 100 mg of amylose (a ratio of 1:10), which is very close to the theoretical calculation that 68 FFA molecules are needed to complex 1 amylose molecule with a degree of polymerization (DP) of 1230. This means the highest molar ratio between amylose and FFA would be 1:68 in a saturated amylose−FFA complex. With regard to the FFA (linoleic acid) in the ternary complex, our previous study on the iodine binding of the ternary complex (*17*) showed a saturation degree of 47% for linoleic acid in the complex, and when this is taken into consideration, the molar ratio of amylose/FFA in the complex could be estimated to be $1{:}(68 \times 0.47) \approx 1{:}32$.

When the ternary complex was formed under the optimum condition (0.68 mg/mL of amylose, 0.25 mg/mL of FFA, and 0.05 mg/mL of whey protein) (*18*), it was found that about 40% (integrated area) of the whey protein participated in complexation [by estimating the amount of the complexed protein peak compared to the total combined protein peaks as observed at UV 280 nm (**Figure 3**)]. Comparatively, 93% of the total supernatant amylose was present in complex through measuring the total carbohydrate content (phenol sulfuric acid assay) of the collected ternary complex fractions. Because β-lactoglobulin (M_w = 18 300 for monomer), which is the major protein component present in the complex, often exists in a dimer form, the molar ratio of whey protein (represented by β-lactoglobulin) and amylose was calculated on the basis of 10 mL reaction volume.

Whey protein:

$$\text{amount of } \beta\text{-lactoglobulin} = 0.5 \text{ mg} \times 0.4$$
$$= 2 \times 10^{-4} \text{ g (40\% of the whey protein is in the complex based on the above data)}$$

$$\text{molecular weight of } \beta\text{-lactoglobulin (dimer)} = 36600 \text{ g/mol}$$

$$\text{number of moles of } \beta\text{-lactoglobulin} = 2 \times 10^{-4} \text{ g}/36600 \text{ g/mol} = 5.46 \times 10^{-9}$$

Amylose:

$$\text{amount of amylose} = 6.8 \text{ mg} \times 0.93 = 6.324 \times 10^{-3} \text{ g}$$

$$\text{molecular weight of amylose} = 200000 \text{ g/mol}$$

$$\text{number of moles of amylose} = 6.324 \times 10^{-3} \text{ g}/200000 \text{ g/mol} = 3.16 \times 10^{-8}$$

Therefore, the ratio of protein/amylose is $5.46 \times 10^{-9}{:}3.16 \times 10^{-8} \approx 1{:}6$.

The final molar ratio among amylose, whey protein [β-lactoglobulin (dimer)], and FFA is 6:1:192, respectively, when the molar ratio results of FFA/amylose and protein/amylose were combined together. Note that possible interactions between whey protein and FFA were omitted because there is only one primary site for β-lactoglobulin to bind FFA (*19*) and, considering that their binding ratio is 1:1, the overall binding of FFA to protein is negligible compared to FFA bound by amylose. A final molar ratio of 6:1:192 for amylose, protein, and FFA, respectively, means that a theoretical value of 4.2% FFA (w/w, linoleic acid as an example) could be bound by the complex. On the basis of the recommendation of ω-3 long-chain fatty acids (1 g/day) by the American Heart Association for those who have coronary heart disease (*20*), the ternary complex, if used as a ω-3 fatty acid delivery system, would be capable of delivering the required amount by the recommendation.

Effect of Monoglyceride and Long-Chain Alcohol on the Formation of the Complex. The fact that amylose and protein rarely interact with each other because of their inherent thermodynamic incompatibility suggests FFAs are critical to the formation of the ternary complex. We previously showed that the aliphatic tail of FFA was complexed with amylose in the ternary complex and that the amylose−FFA inclusion complex was one structural constituent of the ternary complex (*8*). To further investigate the function of FFAs and more specifically their carboxyl headgroup in the self-assembly of the ternary complex, structurally similar molecules

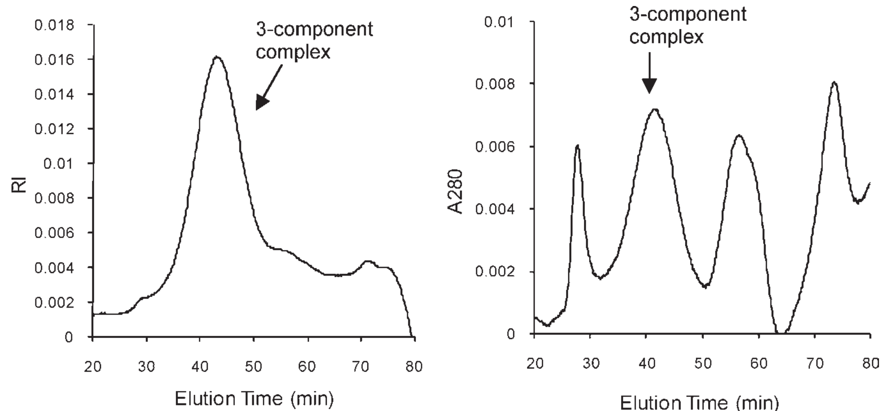

Figure 3. HPSEC profile (RI and UV signals) of the three-component complex. The area under the RI profile was calculated to estimate the amount of complex purity. The area under the UV profile was calculated to estimate the amount of whey protein participating in complex formation.

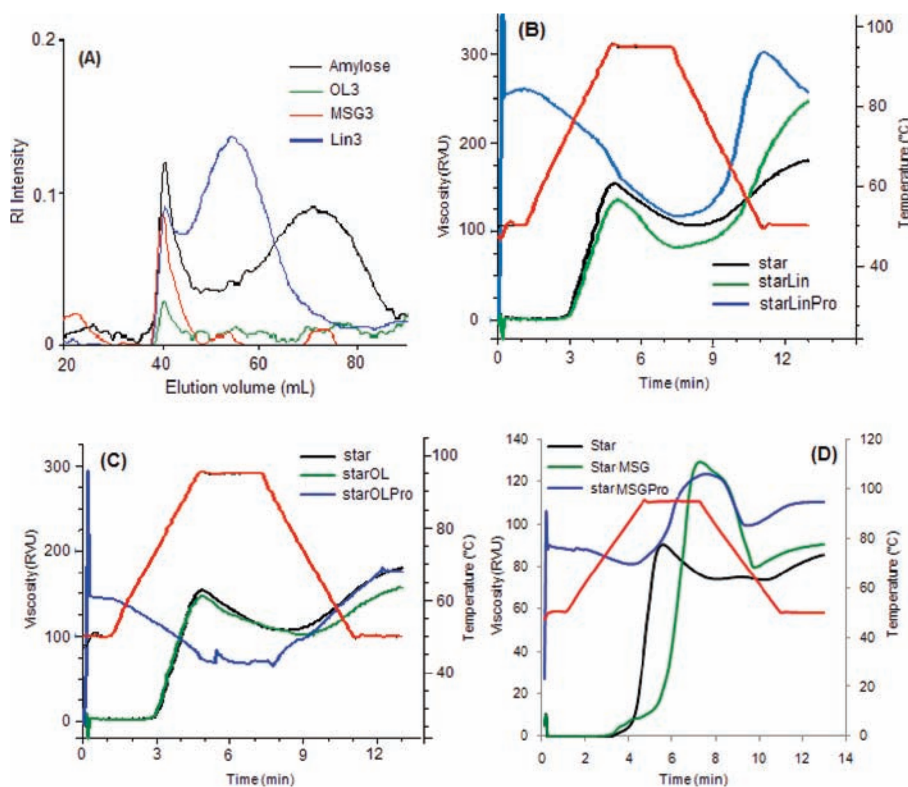

Figure 4. Effect of monoglyceride and long-chain alcohol on the formation of the ternary complex. (A) HPSEC profile when FFA is replaced by cetyl alcohol (OL3) and monostearylglycerol (MSG3). Lin3 is the complex formed with linoleic acid. (B) RVA profile of the starch with linoleic acid and whey protein. (C) RVA profile of starch with cetyl alcohol and whey protein. (D) RVA profile of starch with MSG and whey protein.

of monoglyceride and long-chain alcohol were used with the same amylose and protein samples in the following studies.

No ternary complex was formed when FFA was replaced by either MSG or cetyl alcohol (**Figure 4**). Likewise, there was a loss of the peak representing the ternery complex in the HPSEC profile (**Figure 4A**). For rheological property measurement, the cooling stage viscosity peak (**Figure 4B**) that has been previously demonstrated (*5*) when the three components of FFA, starch, and protein were combined together disappeared when the FFA was replaced by cetyl alcohol (**Figure 4C**) or MSG (**Figure 4D**).

These results demonstrate that the carboxyl group of FFA is essential to the self-assembly of the ternary complex, because the major structural difference between MSG or cetyl alcohol and FFA is their headgroup. Chemically, the pK_a of R−COOH is ~4.5, which means FFA is predominately negatively charged as

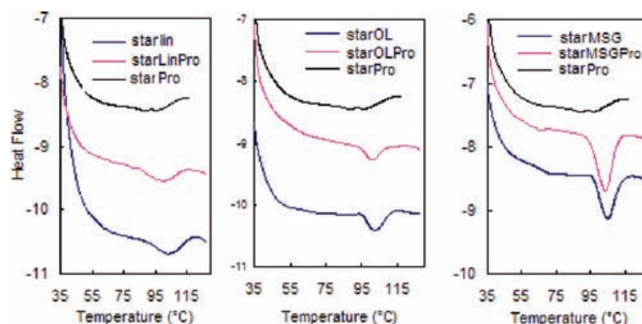

Figure 5. DSC (10 °C/min heating rate) profiles of amylose−lipid complexes formed when linoleic acid (Lin), cetyl-alcohol (OL), and monostearylglyceride (MSG) were used as the lipid components. Pro = whey protein.

R−COO$^-$ at neutral pH. However, no charge (deprotonation) would be produced from hydroxyl R−OH groups (pK_a = 15) that are the headgroup of long-chain alcohol and MSG. This difference in chemical property is apparently the reason for the formation of the complex when FFA with the carboxyl group is used in the system around neutral pH.

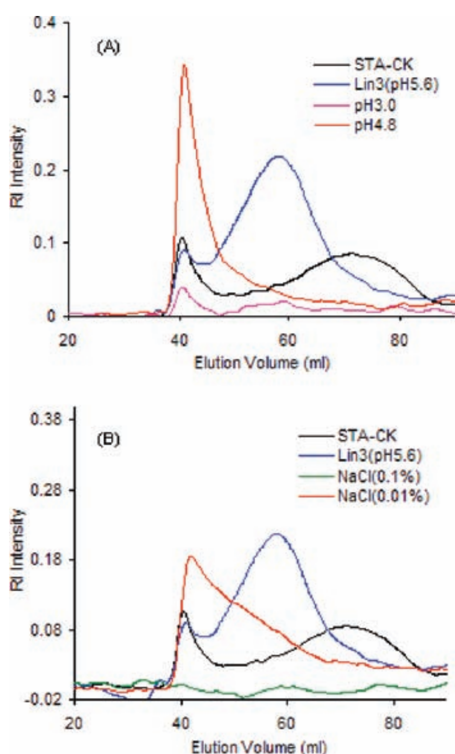

Figure 6. Effect of pH and salt on the formation of the ternary complex during the heating and cooling process. (A) pH and (B) salt. STA-CK, amylose control; Lin3, ternary complex using linoleic acid as the FFA component.

The amylose−lipid complex is formed through the hydrophobic interactions between the aliphatic tail of lipids and the interior of amylose helices, and accordingly, an extensive complexation between amylose and MSG or cetyl alcohol was shown by DSC measurements (**Figure 5**). The DSC peak around ∼105 °C is typical of amylose−lipid complexes (*21*), and the higher values of melting enthalpy of complexes formed with either MSG or cetyl alcohol compared to those with linloleic acid indicate the extent of the complex. The aggregation of the amylose−lipid complex normally causes it to precipitate from solution and, in this experiment, resulted in neither an amylose signal nor cooling stage viscosity peak when MSG or cetyl alcohol was used as the lipid component (**Figure 4**). Consistently, the addition of protein in these systems did not significantly affect the formation of the amylose−lipid (MSG or cetyl alcohol) complex, as evidenced by either the enthalpy or temperature of complex melting (**Figure 5**). This observation indicates that the contribution of protein to the complex formation requires the presence of FFA in the system. In the context of self-assembly of these components, the interactions between the carboxyl group of FFA and polyionic protein molecules may also be essential to the formation of the ternary complex.

Effect of Salt and pH on the Formation of the Ternary Complex. The foundation of the self-assembly of these soft matters to form versatile supramolecular structures is the helical structure of amylose with its large number of hydroxyl groups, the polyionic property of protein, and the amphiphilic attribute of FFAs. The hydrophobic interaction between amylose and the aliphatic tail of FFA was previously proven to be one important force in the ternary complex (*8*). The critical role of the carboxyl group of

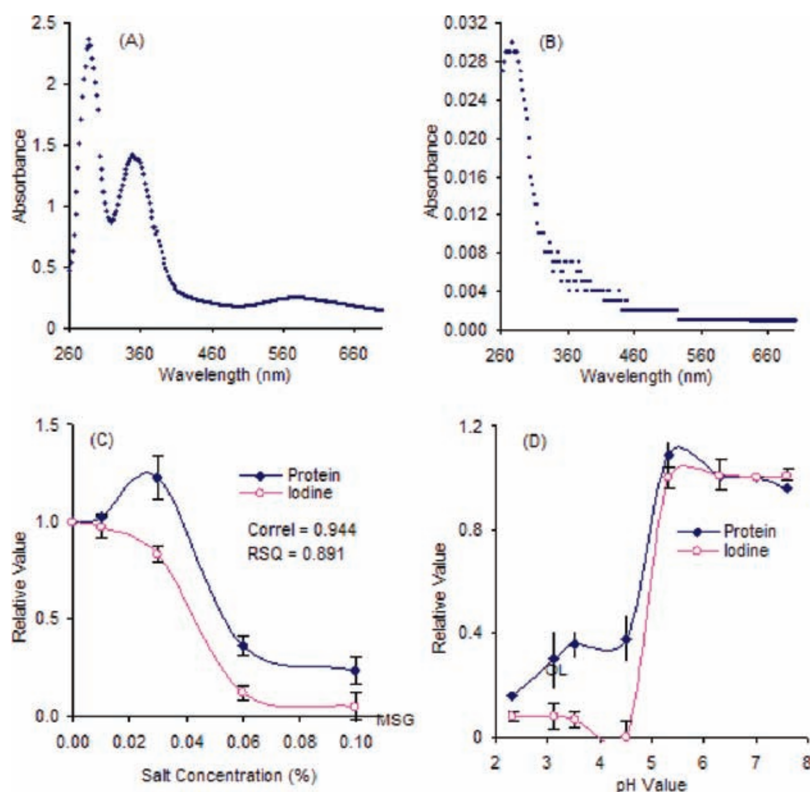

Figure 7. Effect of salt and pH on the stability of the formed ternary complex. (A) UV−vis scanning of the pure complex when iodine solution (KI−I_2) is added. (B) UV−vis scanning of the pure complex solution. The relative protein concentration and OD_{620} of the supernatant change as the (C) salt concentration and (D) pH value increase.

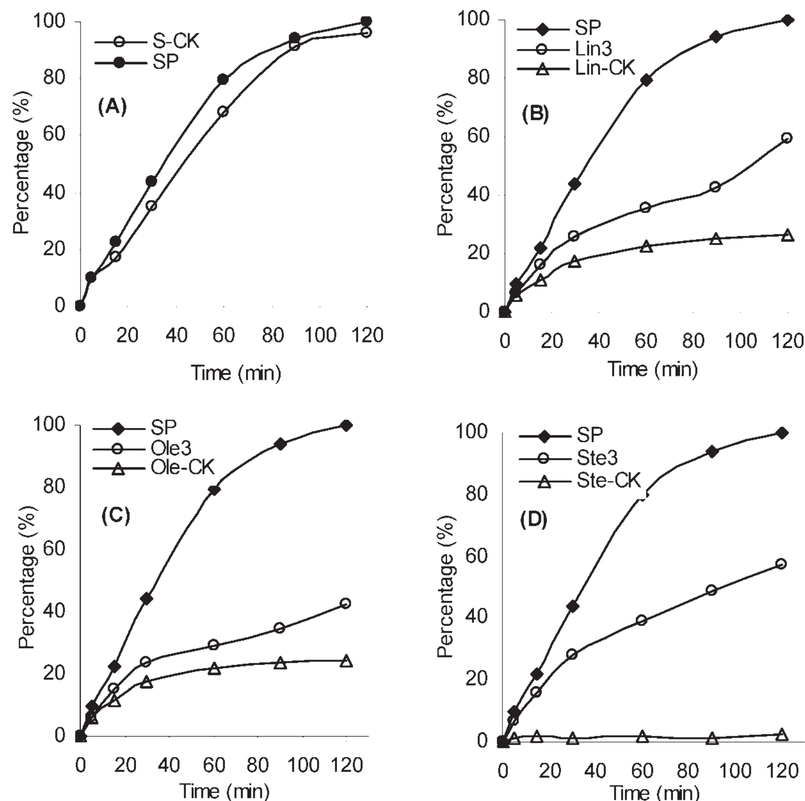

Figure 8. Susceptibility of the ternary complex to α-amylase compared to the FFA−amylose complex and free amylose in solution. S-CK, amylose control; SP, amylose + whey protein; Lin3, ternary complex made with linoleic acid; Lin-CK, amylose linoleic acid. Similarly, Ole3, Ole-CK, Ste3, and Ste-CK are for the ternary complex and FFA−amylose complex made with oleic and stearic acids, respectively.

FFA in the formation of the complex indicates that the electrostatic interaction (the negative charge from carboxyl groups of FFA) might be another important noncovalent force involved in the self-assembly of the nanocomplex. Thus, salt and pH were used as a tool to determine the role of the noncovalent ionic force in the self-assembly of the ternary complex.

When pH was decreased or the salt concentration was increased before the heating and cooling process, both the corresponding ternary complex and amylose disappeared from the HPSEC elution profile (**Figure 6**). In the meantime, the protein concentration in the supernatant (data not shown) did not show parallel changes. Because the protein is one component of the ternary complex, the disappearance of the amylose peak from the HPSEC profile suggests that the amylose−FFA complex was formed and precipitated from the solution under conditions of low pH or the addition of salt. Thus, the formation of the ternary complex was prevented when the charge density of the carboxyl group ($pK_a \cong 4.5$) of FFA was reduced under the used conditions (low pH and salt), which indicates that the negatively charged carboxyl group of FFA is important for the self-assembly of the complex. As the counterpart of electrostatic interaction, the polyionic protein should also be essential to the self-assembly of the ternary complex.

When pH was decreased or the salt concentration was increased after the formation of the ternary complex, a precipitate appeared but each individual component still binds together without dissociation (**Figure 7**). The UV−vis spectrometry scanning results showed a peak at 280 nm that is characteristic of the protein (panels **A** and **B** of **Figure 7**), at 360 nm because of iodine solution, and another at 580 nm for iodine binding by the ternary complex (**Figure 7A**). Similar patterns were found for the relative amount of both protein (1.0 when no salt or pH is changed) and iodine binding (OD_{620}, representing the ternary complex, and the original value as 1.0) in the supernatant as changes of pH or salt concentration (panels **C** and **D** of **Figure 7**), which showed that the protein component always follows the ternary complex and no dissociation occurs. Thus, once the ternary complex is formed, it is stable to salt and pH changes, indicating that the electrostatic functional groups of the ternary complex are also responsible for its solubility in water.

Thus, the ionic interaction is not only important for the formation of the complex, it also makes the complex water-soluble. The carboxyl functional group of FFA is important, and its negative charges are critical for the self-assembly of the complex. The negatively charged FFA is likely the bridge between amylose and protein in the complex.

Enzyme Digestibility of the Ternary Complex. If the ternary complex was used as a delivery system for functional lipophilic compounds (e.g., ω-3 fatty acids), the release property of the carried ligand molecules needs to be known to control its delivery efficiency. In the current study, the amylolytic susceptibility, which is directly associated with the release property, was studied because amylose is the major component of the complex. An intermediate amylolytic digestion pattern between free amylose and amylose−FFA complexes was shown for the ternary complex (**Figure 8**). For the three FFAs tested (linoleic, oleic, and stearic acids), the ternary complexes showed a higher susceptibility to enzyme digestion than the typically hard to digest amylose−FFA complexes. Little digestion of the stearic acid−amylose complex is likely due to the fact that stearic acid has a linear alphatic tail that easily forms a tight complex with amylose, which is consistent with the literature reports that the conformational changes of amylose from random coil to helix induced by complexation reduce the susceptibility to enzymatic hydrolysis (*22*). The intermediate enzyme susceptibility of the ternary complex suggests that there is a proportionally lower amount of amylose

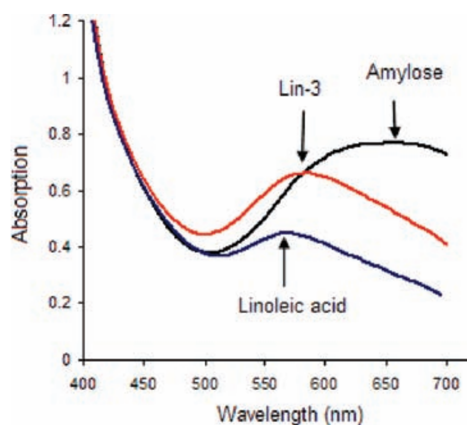

Figure 9. Shift of the wavelength (λ_{max}) with the maximum absorbance when iodine solution was mixed with solutions of the ternary complex compared to free amylose and the amylose−linoleic acid complex.

participating in the inclusion complex or possibly in the helix conformation in the ternary complex than that in the typical amylose−FFA complex. This intermediate enzyme susceptibility, along with an intermediate λ_{max} of iodine binding (**Figure 9**), further supports our previous findings of an "in-between" property of the ternary complex measured by potentiometric titration (*17*). In the meantime, this intermediate digestion pattern also indicates that the carried molecules could be released in a controlled manner if the ternary complex were used as a delivery system for lipophilic compounds in foods.

Self-assembly of the Ternary Complex. Negatively charged FFA is the bridge between amylose and protein in the ternary complexation. The existence of the amylose−FFA complex in the ternary complex (*8*) implies that it is the aliphatic tail of FFA that is connected with amylose, while the negatively charged carboxyl functional group of FFA most likely accounts for the connection with the polyionic protein because amylose is a neutral molecule that would not contribute to the ionic interactions. The disulfide linkages formed between free cysteine 121 of β-lactoglobulin molecules under heat condition may also likely contribute to the formation of the complex, as previously showed that the protein molecules were its organizer (*6*). However, how protein is connected with amylose via FFA during the self-assembly process of the three components needs further investigation because protien is also negatively charged under a neutral pH condition, which would repulse the negatively charged amylose−FFA complex. The local ionic environment of protein might need to be focused on in the future investigations to understand how exactly the protein component contributes to the self-assembly of the ternary complex.

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