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Iodine binding property of a ternary complex consisting of starch, protein, and free fatty acids

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ABSTRACT

A ternary complex consisting of amylose, whey protein, and free fatty acids (FFA) has been identified in our previous investigations, and its iodine binding properties were investigated. After reaction with iodine solution, an absorption peak (λ_{max}) at 620 nm was shown for pure amylose whereas the λ_{max} decreased to 510 nm when amylose was first complexed with FFA. Interestingly, a λ_{max} of 550 nm with an intermediate absorbance was observed for the ternary complex indicating its intermediate spectrophotometric property. Consistently, the amount of iodine bound by the ternary complex was between free amylose and typical amylose–FFA complex from potentiometric titration indicating the amylose–FFA complex within the ternary complex is less compact and more space is left for iodine binding. This in-between property of the ternary complex suggests it can be used as a molecular carrier to accommodate a forth component in addition to its functional lipids carrying capability in food product development.

1. Introduction

A three-component complex formed by starch (amylose), protein and free fatty acids (FFA) after a heating and cooling process was identified previously by our group through rheological property measurement and high performance size exclusion chromatograph (HPSEC) analysis. The rheological property (Zhang & Hamaker, 2003b) using a Rapid Visco-analyzer (RVA) showed an atypical cooling stage viscosity peak in the RVA profile of starch paste in the presence of whey protein and FFA, and the HPSEC chromatogram of a dilute model system (Zhang, Maladen, & Hamaker, 2003a) revealed a distinct ternary complex with a molecular weight of ~6–7 $\times$ 10^6 Da and amylose was the major functional starch molecules in the complex. Further experimental results using different cereal flours (Zhang & Hamaker, 2005) showed similar rheological properties indicating the three-component interaction among starch, protein and FFA might be a common phenomenon in multi-component food systems.

Amylose is an essentially linear macromolecule composing of α-1,4-linked glucosyl units, and it readily binds iodine ions to form a blue complex, which is commonly used to measure the content of amylose (Knutson, 1986) spectrophotometrically and/or potentiometrically. For spectrophotometric method, there are variations in terms of absorptivity and wavelength of the maximum absorbance (λ_{max}) depending on the degree of polymerization (DP) (Bailey & Whelan, 1961) and the structural properties of amylose molecules, which determine the composition of the polyiodine chains complexed with amylose helices. Yu, Houtman, and Atalla (1996) demonstrated that the primary polyiodide chains were composed of I_3^- and I_5^- subunits, and they could be combined to form four dominant polyiodide chains (I_9^{3-}, I_{11}^{3-}, I_{13}^{3-} and I_{15}^{3-}) with different absorbance spectra when complexed with amylose. Overlapping of these different polyiodine chains produces the characteristic spectrum of the amylose–iodine complex. Additionally the length of the α-1,4-linked glucosyl units available for amylose-iodine complex formation influences the relative proportions of the individual spectrum. Potentiometric titration of iodine is another method that directly measures the amount of bound iodine independent of the length or properties of the amylose in samples. Thus, a combination of spectrophotometric measurement and potentiometric titration will give a better description of iodine binding properties of the amylose molecule, and help to understand the molecular structure of amylose under different conditions.

The property of amylose–iodine complexation has been used in our previous studies showing amylose is the major starch molecule participated in the ternary complex. Differential Scanning Calorimetry (DSC) analysis of the ternary complex revealed that amylose–FFA complex was the major secondary structural component in the ternary complex, and its decreased melting enthalpy compared to

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typical amylose–FFA complex (Zhang & Hamaker, 2004) indicated that the iodine binding by the ternary complex would be different from typical amylose–iodine complex. Therefore, a detailed characterization of the iodine binding properties of the ternary complex is necessary to further the understanding of the structure of the ternary complex formed during the three-component interactions.

2. Experimental

2.1. Materials

Normal maize starch was defatted with 85% methanol for 16 h at room temperature before experiment. Whey protein isolate (WPIBioPro, 97.5% protein) was from Davisco Foods International, Inc. (Eden Prairie, MN). Lauric (C12:0), myristic (C14:0), palmitic (C16:0), stearic acid (C18:0), and linoleic (*cis*-9,*cis*-12-octadecenoic acid, C18:2) acids were from Sigma Chemical Co. (St. Louis, MO). Double distilled water was used in the experiment.

2.2. Amylose, protein and FFA complexation in a dilute system

A dilute system was used to examine amylose–protein–FFA complexation. The normal maize starch solution (0.5%) was prepared by heating for 20 min in a boiling water bath and then cooled to room temperature. After centrifugation at 30,000*g* for 20 min, the supernatant was used as the amylose solution for interacting with protein and FFA. Interaction among amylose, FFA, and protein was performed by adding 5, 2.5, 1.25, 0.5 mg of whey protein (dissolved in purified water, 10 mg/mL) and 2.5 mg of FFA (dissolved in petroleum ether, 10 mg/mL) to a capped glass tube containing 10 mL amylose solution and then cooked for another 20 min in a boiling water bath with continuous stirring. When the cooked sample was cooled slowly overnight at room temperature with continuous stirring, the supernatant was filtered through a 0.45 μm filter and injected into a HPSEC-MALLS system (Zhang, Zihua, & Hamaker 2006) for analysis. For comparison, amylose–FFA complex was also prepared by adding corresponding volume of water (compensating the volume of protein solution), and amylose–protein mixture were also kept the same volume by adding water.

The optimum condition to form the three-component complex was found to be 0.5 mg whey protein and 2.5 mg FFA in 10 mL normal maize starch solution. The differential scanning calorimetry (DSC) analysis of the freeze-dried materials used in the dilute model system was performed according to the procedure of Zhang et al. (2006).

2.3. Reaction between iodine and the ternary complex

Iodine solution (2% KI, 0.2%I_2) stored in a nonactinic bottle was used as the standard iodine solution to react with the prepared ternary complex. For comparison, amylose–FFA complexes were also prepared in the same way without adding protein. Then the solutions (2 mL, with the same amylose concentration) of amylose, amylose + whey protein, amylose–FFA complex, and the ternary complex prepared following the above procedure were reacted with 0.2 mL iodine solution. The UV–Vis spectra of the samples were measured using a UV–Vis spectrophotometer Model TU-1900 (Peaking Puxi Inc. Beijing) from 200 to 800 nm.

2.4. Potentiometric titrations of the bound iodine by the ternary complex

A digital pH/mV meter was used (Corning pH/ion analyzer 350, Corning, NY) to measure the iodine binding ability of the samples. The electrode was a platinum redox combination electrode, having an epoxy body with replaceable junction and refillable electrode with a BNC connector (Corning, Cat. No 476516, Big Flats, NY). The amylose solution (10 mL + 50 μL water), amylose–FFA complex (10 mL amylose + 2.5 mg FFA + 50 μL water), and a dilute system of starch–protein–FFA (10 mL amylose + 50 μL protein + 2.5 mg FFA), which were prepared following the procedure described before, were used as the samples for iodine titration. After being filtered through a 5 μm filter, the samples was titrated potentiometrically with iodine as described by Schoch (1964).

Sample solution (5 ml) and 2.5 ml of 0.4 N potassium iodide (KI) solution were stirred in a beaker at a constant rate with a magnetic stirrer. Successive amounts of potassium iodate (KIO_3) were added gradually, and change in millivolts was read at increments between 240 and 290 mV. A blank containing 5 ml distilled water and 2.5 ml 0.4 N KI was used as a control. From the mV readings, the concentration of free iodine (I_3^-) in the solution was determined using a calibration curve. The bound iodine was estimated from the difference between the total amount of iodine added to the model system and the free iodine at each point of the curve. Bound iodine (Y) was then plotted against free iodine (X).

3. Results and discussion

3.1. HPSEC analysis of the ternary complex

HPSEC is the method used before to detect the formation of the ternary complex consisting of starch, protein and FFA, but the ternary complex peak was not so clear (Zhang et al., 2003a). After optimizing the relative content of whey protein component, a clear and distinct peak was shown in the HPSEC profile after a heating and cooling process (Fig. 1 top, Lin3) when linoleic acid was used as the FFA component. The major component of the initial starch solution after centrifugation was amylose with a broad distribution (Fig. 1, starch-CK) and residual amylopectin represented by a small peak. The disappearance of 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 including oleic acid and stearic acid were used as the FFA component (Fig. 1 bottom, CK-SPR is the HPSEC profile of starch + whey protein). Therefore, the ternary complex formed among amylose, whey protein and FFA can be easily detected using a technique of HPSEC.

3.2. Iodine binding of the ternary complex measured by potentiometric titration

Potentiometric titration offers the most definitive measure of the iodine binding capacity of amylose independent of the structural changes of the amylose molecules under different conditions. Literature reports (Kim & Robinson, 1979; Yamamoto, Sano, Harada, Yasunaga, & Tatsumoto, 1984) have shown that the presence of FFA or surfactants, which formed amylose–lipid complex (Godet, Bizot, & Buleon, 1995), decreased iodine affinity to amylose as revealed by potentiometric measurement. Our results of amylose–FFA complex with a lower iodine binding compared to free amylose (Fig. 2) is consistent with these observations. Less available space in the helical cavity of amylose complexed with FFA is generally considered as the cause for the lower iodine binding capacity of the amylose–FFA complex.

The amylose–FFA–protein ternary complex showed an intermediate iodine binding property between free amylose and typical amylose–FFA complex as shown in Fig. 2. The relative iodine affinity of 100.0%, 53.0%, and 14.5% for free amylose, ternary complex and amylose–FFA complex, respectively, were calculated based

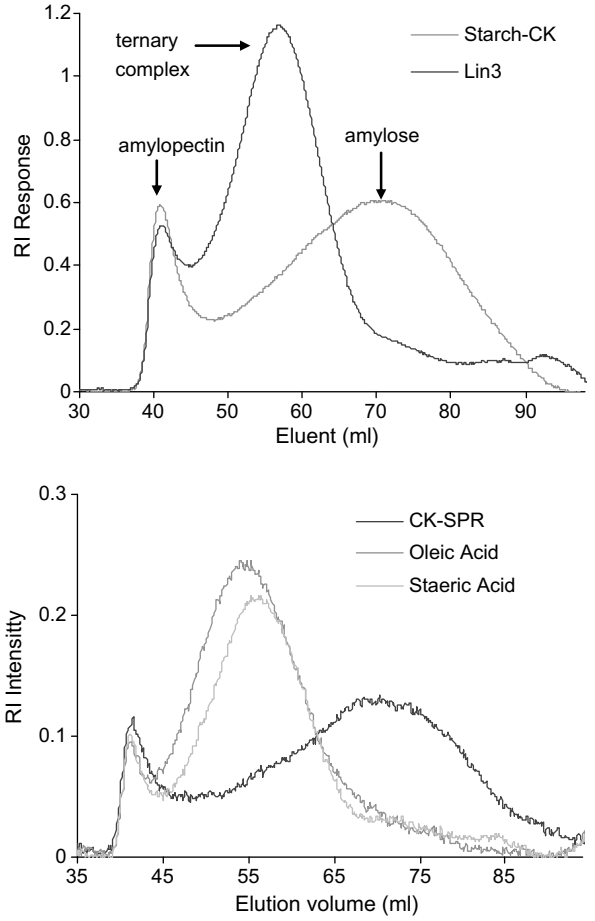

Fig. 1. Typical ternary complex consisting of amylose, whey protein, and FFA of linloleic acids (top), stearic and oleic acids (bottom). Starch-CK: starch control solution, Lin3: the ternary complex when the FFA component is linloleic acid. CK-SPR: starch + whey protein control.

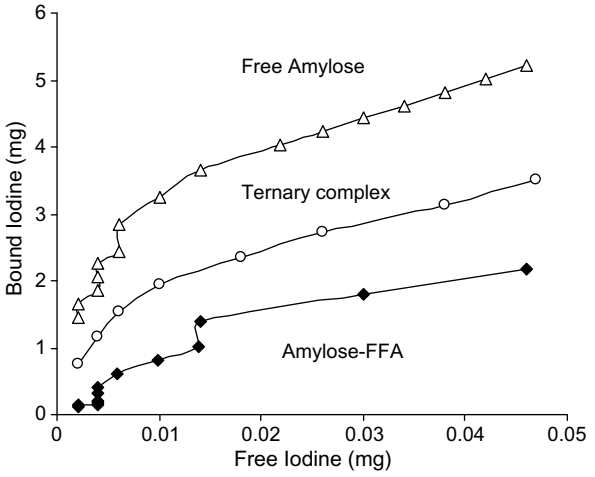

Fig. 2. Iodine binding measured by potentiometric titration. The extrapolation points represent the iodine binding capacity of each sample with the same amount of amylose.

on regression analysis using the data points in the up linear portion. We have shown that the amylose and FFA complex in the ternary complex, with a decreased melting enthalpy, is different from the typical amylose–FFA complex (Zhang & Hamaker, 2004), and a lower value of melting enthalpy means less amount of V-type amylose–FFA crystallites and more empty space in the amylose helical cavity that is available for iodine binding.

Based on the literature study of typical amylose–FFA complexation (*Murdoch, 1992*), the helical structure of amylose is the foundation for amylose–FFA complexation, and it needs three turns of amylose helix (six glucosyl units per turn) to complex with one FFA molecule. Although we have identified that amylose–FFA complex is one important structural element of the ternary complex (Zhang et al., 2003a), a higher value of the iodine binding by the ternary complex than typical amylose–FFA complex suggests the FFA in the ternary complex is not tightly packed in amylose cavity as it does in a typical amylose–FFA complex, which is evidenced by decreased enthalpy of melting at ~100 °C (Table 1). The high iodine binding is probably caused by the 'special' amylose–FFA complex in which fewer glucosyl units are needed to wrap FFA molecules and/or amylose is partially accessible to FFA, and in either case, there would be more space in the helical cavity of amylose for iodine binding. The exact reason for the 'special' amylose–FFA interaction in the ternary complex could be explained by the competition of FFA molecules between amylose and whey protein, but the FFA used in the system is way above the concentration saturating both amylose and whey protein (there is still FFA left after the ternary complex formation based on observation of a film-like non-aqueous top layer). How the presence of whey protein makes this 'special' amylose–FFA interaction with a higher value of iodine binding needs further investigation.

3.3. Iodine binding of the ternary complex measured by spectrophotometric method

Potentiometric titration results have given the definitive iodine binding value of the complex, but the structural base for the in-between iodine binding properties of the ternary complex is elusive since the method itself is independent of the molecular structure of the amylose. Thus, spectrophotometric properties of the ternary complex including the absorptivity and the wavelength of the maximum absorption (λ_{max}) were used to further characterize the structural properties of the ternary complex.

The spectrophotometric measurement showed that the amylose–iodine reaction in the addition of whey protein, especially at higher concentrations, had a decreased absorptivity (Fig. 3B) after cooking process although we showed before that there was no interactions between amylose and protein (Zhang et al., 2003a; Zhang and Hamaker, 2003b). Apparently, the cooking process was necessary to the observed results since a simple mixture of amylose and whey protein did not noticeably affect the iodine affinity of the amylose (Fig. 3A). We had shown that a reducing agent of 2-mercaptoethanol treatment disrupted the ternary complex by cleaving the disulfide bond linked whey proteins (Zhang et al., 2003a), and so the cooking process was necessary for the whey protein to link each other into large aggregates. The linked whey protein might interact with amylose through physical mu-

Table 1
DSC results of freeze-dried starch material used in the dilute model system

Samples	Peak Temperature (°C)	Enthalpy (ΔH, J/g)
S*	102.3 ± 0.10	1.19 ± 0.19
S + Staeric acid	101.7 ± 1.03	17.20 ± 0.34
S + Staeric acid + W*	104.3 ± 0.98	8.87 ± 0.41
S + Oleic acid	99.1 ± 1.64	14.78 ± 0.36
S + Oleic Acid + W	101.1 ± 1.48	8.28 ± 0.51
S + Linoleic Acid	95.1 ± 2.14	10.12 ± 0.61
S + Linoleic Acid + W	95.4 ± 2.82	8.21 ± 0.78

*S: normal maize starch used in the dilute model system after centrifugation.
*W: whey protein isolate.

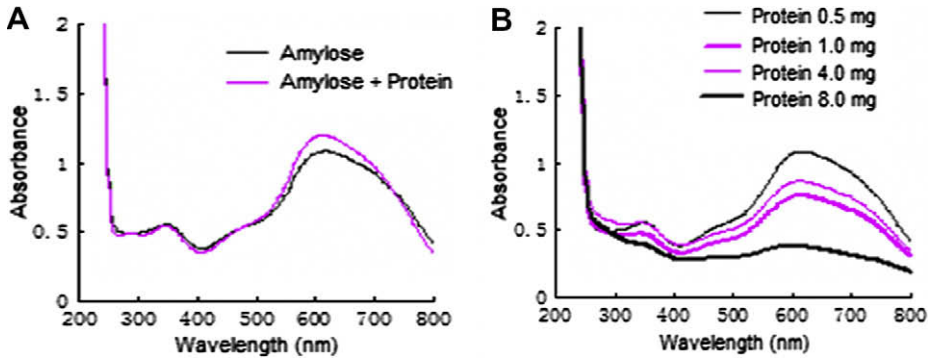

Fig. 3. The UV–Vis scanning spectrum of amylose–iodine complex in the presence of whey protein (A) without heating (0.5 mg protein), and (B) after heating/cooling process with different amounts of protein in 10 mL amylose solution.

tual entanglement to affect iodine binding. On the other hand, the relatively constant λ_{max} at different protein concentrations indicates that their interactions have no effects on the unit chain length of the amylose that is available to complex with iodine since the λ_{max} is length dependent in a certain range of DP (Pfannemüller, Mayerhöfer, & Schulz, 2004). Overall, whey protein in the three-component complex also plays a role in the iodine binding property of the ternary complex.

The iodine affinity to the typical amylose–FFA complex was noticeably decreased as shown in Fig. 3 with a much lower absorptivity (by comparing Fig. 3 and Fig. 4), which was consistent with the potentiometric titration results. Additionally, the λ_{max} also moved from 620 nm to ~510 nm indicating the unit chain length of amylose for amylose–iodine reaction was decreased by the FFA complexation. Therefore, both the absorptivity and λ_{max} of the amylose–iodine complex were affected by FFA complexation.

The iodine–ternary complex interaction showed, the same as the potentiometric results, an intermediate property between free amylose and typical amylose–FFA complex in terms of both absorptivity and, particularly, λ_{max} (Figs. 5 and 6). Similarly, the heating/cooling process is necessary for the three component interaction to form a ternary complex, and the addition of high concentration of the protein also decreases the absorptivity without affecting the λ_{max} (Fig. 5). It is known that the absorptivity is amylose-concentration dependent and the λ_{max} is chain length dependent. The lower absorptivity and lower value of λ_{max} compared to free amylose suggests the accessibility of amylose to iodine binding is decreased and the DP of accessible amylose chains is also reduced. These reductions suggest that the distribution pattern of FFA molecules in the ternary complex could result in shorter empty amylose chains for iodine to bind and thus a reduction of λ_{max}. In the meantime, the further decreased absorptivity and λ_{max} of the typical amylose–FFA complex compared to the ternary complex further strengthen the intermediate property of the ternary complex concerning its 'special' amylose–FFA complex in the presence of whey protein.

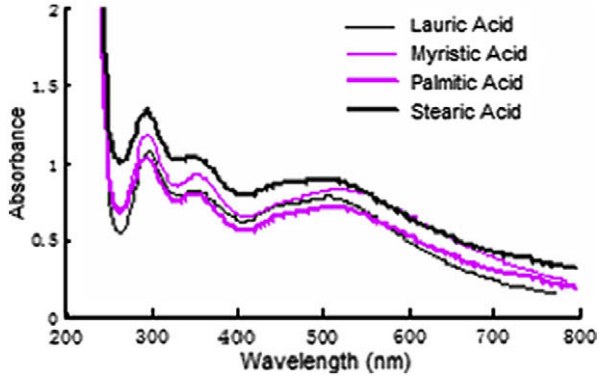

Fig. 4. The UV–Vis scanning spectrum of amylose–iodine complex when different FFA was first complexed with amylose.

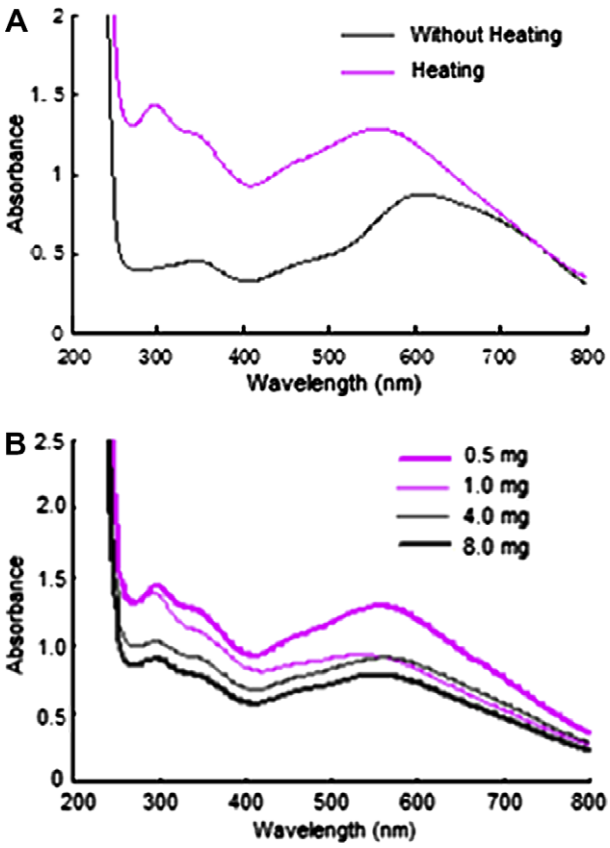

Fig. 5. The UV–Vis scanning spectrum of the ternary complex. (A): The comparison between heating and without heating process (0.5 mg protein), (B) The effects of different amounts of whey protein.

3.4. The intermediate iodine binding properties and the function of protein

Both potentiometric titration and photometric measurement demonstrated the in-between iodine binding properties of the ternary complex. Compared to typical amylose–FFA complexation, the presence of whey protein did change the dynamics of amylose and FFA interaction. Our previous result of an increased exothermic enthalpy of the amylose–FFA complexation (Zhang & Hamaker

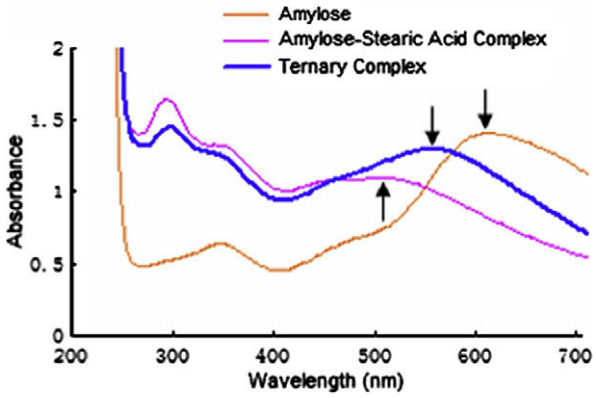

Fig. 6. The UV–Vis scanning spectrum of ternary complex made from amylose, whey protein and stearic acid. The arrows represent the λ_{max} for each model system with different combinations of amylose, whey protein, and FFA.

2004) in the ternary system compared to typical amylose–FFA complexation also reflects the dynamics of the amylose and FFA interaction in the presence of protein.

The effect of heating process signifies the importance of whey protein cross-linking, the ternary complex formation, and the special amylose–FFA complexation. Whey protein itself could decrease the absorptivity at a higher concentration, but for the used concentration (0.5 mg/10 mL), the effect is not significant (Fig. 3), thus, the cross-linking to form large molecules and to entangle with amylose is likely more important. β-Lactoglobulin, the major component in the whey protein can bind FFA through either hydrophobic or ionic interactions (Perez & Calvo, 1995), and this protein–FFA binding might act as anchor force to orientate (as a drag force to take part of FFA out of amylose helix, so fewer glucosyl units are needed to complex one FFA molecule) and to stabilize this special FFA–amylose complexation to produce a quick formation of the amylose–FFA complex (within the ternary complex as shown by the increased exothermic enthalpy, Zhang & Hamaker 2004) with an in-between property of iodine binding.

The in-between property of iodine binding can also be illustrated from the viewpoint of molecular aggregation as the λ_{max} is related to the degree of molecular aggregation (Cronan & Schneider, 1969). The formed ternary complex is water-soluble and stable in solution (Zhang et al., 2003a), but the typical amylose–FFA complex exists as a cloudy suspension reflecting its higher degree of aggregation. Thus, a random-coil free amylose in solution is the least in aggregation (large value of λ_{max}) while the typical amylose–FFA is highly aggregated (small value of λ_{max}), and the intermediate aggregation of the ternary complex would have an intermediate iodine binding property in both absorptivity and the wavelength of maximum absorption.

4. Conclusion

An intermediate iodine binding property of the ternary complex formed during interactions among starch, protein and FFA was revealed. The intermediate iodine binding properties between free amylose and a typical amylose–FFA complex further signify the specialty of the amylose–FFA complexation in the presence of whey protein, which results in changes of the dynamics of amylose–FFA complexation with an intermediate degree of aggregation. The distribution pattern of FFA molecules in this special type amylose–FFA complex is likely the root cause for the intermediate iodine binding properties of the ternary complex. The presence of FFA in the complex indicates that this ternary complex can be used as a molecular carrier of functional lipids to protect its bioactivity, and more empty space in the ternary complex could also accommodate a forth component. The intermediate property of the iodine binding can also be utilized as a simple and quick method for verifying starch–protein–FFA three component interactions and the formation of the ternary complex.

Acknowledgements

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