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Effect of molecular weight of dextran on the phase behavior and microstructure of preheated soy protein/dextran mixtures

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Abstract

The effects of molecular weight of dextran on the phase behavior and microstructure of preheated soy protein (heat-induced soy protein aggregate)/dextran mixtures have not been reported before. Hereby mixed systems of heat-induced aggregates with different size and dextran with different molecular weight have been investigated at room temperature (25 °C) and pH 7.0. Phase diagrams were established by centrifugation, chemical assays and visual observation. The mixture of dextran with larger molecular weight and larger aggregate phase separated at lower biopolymer concentration. The microstructures of the phase separated mixtures were described using confocal laser scanning microscopy (CLSM), which revealed the association of protein aggregates. The image analysis of the CLSM images showed that the histograms of the gray values were different significantly. Observations of small deformation rheology (G', G'') of the mixed system at concentrations corresponding to those of CLSM measurements provided additional information of the microstructures.

Keywords: Dextran; Aggregate; Phase separation; Confocal microscopy; Rheology

1. Introduction

The phase separation in globular protein and polysaccharide mixtures has been widely studied because of some practical considerations: for example, phase separation determines the texture of processed food (Norton & Frith, 2001; Walkenstrom & Hermansson, 2002).

Native globular proteins and polysaccharides with the same charge may phase-separate from each other at relatively high volume fractions of the components. However, it has been noted that the increase in particle size as a consequence of heat-induced aggregation of protein will enhance demixing (Croguennoc, Nicolai, & Durand, 2001a; Syrbe, 1997). These authors believed that the phase separation is induced by the depletion of the polysaccharides from the region between neighboring protein aggregates.

Heat-induced gelation of soy protein plays an important role in soy food process, which has long been exploited to produce foods with different structural and textural characteristics (Tay, Kasapis, Perera, & Barlow, 2006). Researches on a number of different proteins, including soy proteins, have proposed a multi-stage mechanism for globular protein gelation: thermally induced unfolding is the first step followed by aggregation and cross-linking. Now more and more researchers believed that phase separation introduces a fourth stage in the protein gelling mechanism (Clark, Kavanagh, & Ross-Murphy, 2001). Phase separation is one of the most fundamental phenomena responsible for the formation of heterogeneous structures

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of gels (Gunton, San Miguel, & Sahni, 1983). San Biagio et al. (1999) indicated that demixing is started by the thermodynamic drive toward the formation of protein-rich and solvent-rich regions; phase separation promotes the further formation of the protein aggregates. However, the real process must be more complex and the effects of the protein aggregation on demixing should also be considered. Utzumi, Damodaran, & Kinsella, (1984) proposed that the heat-treatment of soy protein will lead the co-existence of larger aggregates and small protein subunits. A mixture of large and small particles is a typical example of dynamically asymmetric system and phase separation is commonly observed in this system (Tanaka, Nishikawa, & Koyama, 2005). It is very difficult to study the effect of protein aggregation on phase separation in the pure protein system since, as indicated by Clark et al. (2001), the highly kinetically determined non-equilibrium behavior prohibited the determination of phase diagram.

In a paper aimed to study the aggregation, gelation, and phase separation of heat denatured globular proteins, Durand, Gimel, and Nicolai (2002) first investigated the phase behavior of β-lactoglobulin and κ-carrageenan mixed system and derived the conclusion by comparing the similarity in gel microstructure of the β-lactoglobulin–κ-carrageenan system to that of pure β-lactoglobulin gel.

A similar approach was applied in the present study in which a colloid-polymer system was constructed by blending soy protein aggregates with polysaccharides. The phase behavior of the aggregated soy proteins was obtained by making projection from protein–polysaccharide system to pure protein system. It is reasonable by this strategy since in this case the colloids (soy protein aggregates) could be treated as a pseudo one component system, and the polymers (polysaccharides) in solution are considered only through the effective pair potential between the dissolved colloids (Striolo, Colina, Gubbins, Elvassore, & Lue, 2004). Molecular weight of polysaccharide also plays a predominant role in affecting the phase behavior of protein and polysaccharide mixtures (Bourriot, Garnier, & Doublier, 1999a). Low molecular weight will show lower effective pair potential between colloids. Doublier, Garnier, Renard, and Sanchez (2000) found that decreasing the molecular weight of the polysaccharide results in an increase of its concentration needed to obtain demixing of the system. So we chose two kinds of dextran with average molecular weight of 400,000 (DT400) and 5,000,000 (DT5000) and different soy protein aggregates to study phase separation. Dextran is a neutral polymer, and its structure has been well outlined (Tvaroska, Perez, & Marchessault, 1978). In an aqueous solution, dextran is a random coil and not able to form a gel. In our previous researches, we studied the effect of protein concentration on the particle size of soy protein aggregates. Heat-induced aggregates with various particle sizes could be obtained by varying the concentration of soy protein solutions under controlled heating condition (Li et al., 2007). For above purpose, (i) the phase behaviors of these mixtures have been described, (ii) their microstructures have been observed by CLSM at room temperature, and (iii) additional information of the microstructures was obtained from small deformation rheology (G', G'').

2. Materials and methods

2.1. Sample preparation

The dextrans were obtained from Sigma Co. (St. Louis, MO, USA). The solutions were prepared by as follows: the powders were suspended in distilled water and stirred for 2 h at room temperature. The pH was adjusted to 7.0, and 0.02% NaN_3 solution was added to avoid bacterial growth. The final solutions were filtered through cellulose acetate membranes with pore size of 0.45 μm (Merck, Germany) to remove any insoluble particles.

Low-denatured, defatted soy flour was provided by Shandong Gushen Industrial & Commercial Co., Ltd. The flour has protein content of 55.0% ($N \times 6.25$, dry base) and nitrogen solubility index of 87%. All other reagents and chemicals were of analytical grade.

Defatted soy flour, which had been ground to pass 80 meshes, was extracted with 85% aqueous alcohol at 25 °C for 1 h with a ratio of 1:5 of flour to solvent. The slurry was vacuum filtered and the filter cake was extracted with 95% aqueous alcohol again at 25 °C for 1 h with a ratio of 1:2 of flour to solvent. The slurry was also vacuum filtered and the filter cake was vacuum dried at room temperature. The dried material was ground to pass 80 meshes. The obtained soy flour devoid of oxidized lipid induced protein aggregation was suspended in distilled water with a liquid solid ratio of 15:1 and 2 N NaOH was added to adjust pH to 7.0. After stirring for 1 h at room temperature, the suspension was centrifuged at 10,000*g* for 30 min at 4 °C to recover the supernatant. Soy protein was precipitated by adjusting pH to 4.5 with 2 N HCl and centrifuged at 10,000*g* for 30 min at 4 °C. After washing the curd with distilled water, the protein precipitate was re-suspended in distilled water and neutralized to pH 7.0 with 2 N NaOH and kept in refrigerator overnight. After centrifuging at 10,000*g* for 30 min at 4 °C to remove small quantity of insoluble substances, the clear solution was dialyzed at 4 °C for 24 h and then freeze dried and stored at 4 °C. Soy protein prepared from alcohol washed soy flour contained 98.09% of protein, substantially higher than that of soy protein directly from defatted soy flour (conventional soy protein), lower turbidity and the solubility was close to 100%.

Soy protein aggregates with different particle size were prepared by suspending the soy protein in distilled water with concentration of 1% and 5% (wt %) and stirring thoroughly. The suspensions were centrifuged at 10,000*g* for 15 min and the supernatants were filtered through cellulose acetate membranes with pore size of 0.45 μm (Merck, Germany) to remove any insoluble particles. Small soy protein aggregates (SA) were prepared by heating a solution containing 1% soy protein without salt for 15 min at

100 °C, and cooling to room temperature in an ice bath for 5 min. Larger soy protein aggregates (LA) were prepared by heating a solution containing 5% soy protein at the same conditions. The solutions were characterized using size exclusion chromatography and laser light scattering. We have reached the conclusion that the aggregates formed by heating soy protein at pH 7.0 are polydisperse, formed by association of small relatively mono-disperse primary aggregates and have a self-similar structure. The hydrodynamic radius as revealed by unimodal analysis is about 37.0 nm (SA) and 144.9 nm (LA), respectively (Li et al., 2007). Above aggregate solutions were concentrated to certain concentration for phase separation. The aggregates are stable to dilution and may therefore be investigated by various techniques at different stages of the process (Croguennoc, Nicolai, & Durand, 2001b).

2.2. Determination of phase diagrams

Phase diagrams were established based on centrifugation and chemical assays combined with visual observation. A large number of mixtures with different biopolymer (SA, LA and two kinds of dextran) concentrations were prepared. The tie points that linked the compositions of phases in equilibrium were given at the same time. The final binodal line of phase separation was plotted by combining these tie points directly. The gel points of the mixtures were obtained based on the visual observation. A mixture was determined to be a gel when the mixture solution was transformed into a solid (Zhang & Foegeding, 2003). During all the experiments, the concentration of soy protein was determined by Kjeldhal method (AOAC, 1970). The dextran concentration was determined by phenol/sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

Plots of final phase diagrams were performed using Origin 6.0 software (Microcal, USA).

2.3. Microscopical observations (CLSM) and image analysis

Soy protein aggregates were stained by adding fluorescein isothiocyanate (FITC) to the protein solution under magnetic stirring during 1 h and 30 min (Donato, Garnier, Novales, & Doublier, 2005; Schorsch, Jones, & Norton, 1999). Different mixtures with protein content of 4% and polysaccharide content of 1% were prepared as described above and poured between a concave slide and a coverslip, then hermetically sealed. Observations were performed on CLSM with a Leica TCS 4D confocal microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) and a 100 mm oil immersion objective lens. The excitation using an air-cooled Ar/Kr laser was performed at 485 nm.

Gray value distribution of the CLSM images of soy protein aggregate/dextran mixtures based on Matlab 6.5 programming (Math Works Company, USA) were carried out. Each image was transformed to 1000×1000 pixels, with a gray value range from 0 to 255. Images of phase separated mixtures will contain regions with 'high' gray values (white) and regions with 'low' gray values (black). The x-axis of the histogram describes the gray value and the y-axis describes the frequencies that the gray values occur. The histogram of gray values demonstrates the basic statistic feature of the image; describes the numbers of the image pixel of each gray value and indicates the frequencies of each gray value occurred in the image. In the histogram a larger gray value shows a higher frequency column, and reflects that the protein associated parts are much denser (Zhang, Peng, & Wang, 2002).

2.4. Determination of dynamic moduli

Dynamic moduli (G', G'') were obtained from small deformation measurements using an AR1000 controlled stress rheometer (TA Instruments, UK) equipped with a Peltier temperature controller and a flat-plane device (40 mm diameter, 0° angle). The large gap used in this geometry (1 mm) means that the possible interface effects are much reduced. Measurements were made at 1% strain and 1 Hz frequency. The measurement temperature was 25 °C and was controlled within 0.1 by a thermostat bath. The temperature was stabilized in less than 1 min. Time sweeps were carried out over the range of 0–25,000 s. The mixtures were poured onto the flat-plane of the rheometer directly after preparation (i.e., before the onset of phase separation) and covered with a thin layer of paraffin oil to prevent evaporation. Frequency sweeps were followed time sweeps with range changing from 0.1 to 10 Hz at 25 °C.

3. Results and discussion

3.1. Phase diagrams of soy protein aggregate/dextran systems

3.1.1. Phase diagram of soy protein aggregate/DT400 system

The phase diagrams of soy protein aggregate/DT400 mixtures at room temperature are found in Fig. 1. The binodal line (solid curve) in soy protein aggregate/DT400 mixtures divided the mixtures into stable region (compositions below the curve, plus sign) and unstable region (compositions above this curve, open triangle). Increasing the concentration of protein or DT400 initially caused phase separation then, as concentration was further increased, gelation (e.g., gel points, open circle). The tie-lines imparted a high asymmetry of the diagram, i.e., they showed that mixed solutions of these biopolymers separated into phases differing greatly in the concentrations of the two macromolecular components. The results of chemical assays indicated that the segregative phase separation took place. According to the classical Flory–Huggins theory, when the segregative phase separation takes place, the interaction parameter χ is positive, indicating a net repulsion between soy protein aggregates and dextran. This

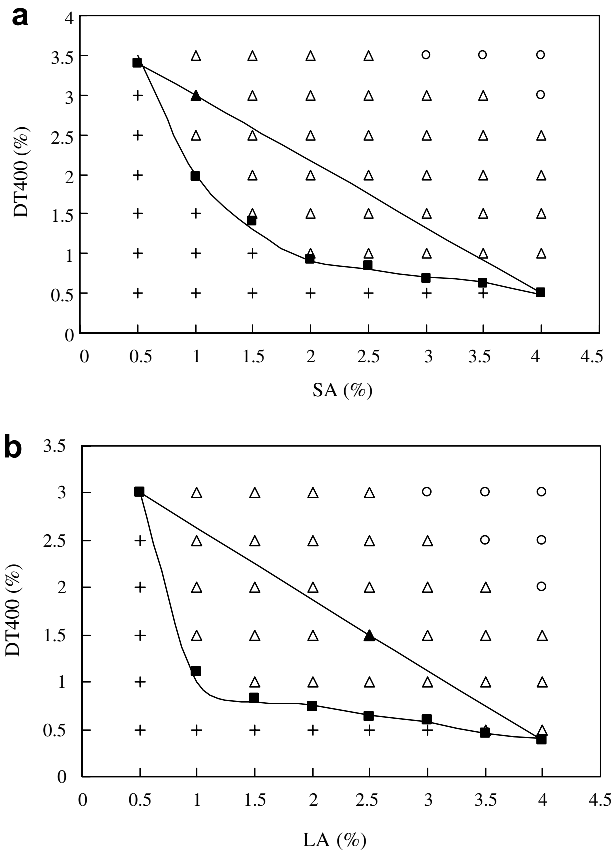

Fig. 1. Phase diagrams of soy protein aggregate/DT400 system established at room temperature, pH 7.0. The solid line is the binodal line of phase separation; ▲, representative initial mixtures of soy protein aggregate/DT400 for chemical analysis; ■, tie points (represent the compositions at upper and bottom phases separated from the initial mixtures, which obtained by chemical assays); +, stable mixtures; △, unstable mixtures; ○, gel points. (a) Small aggregate (SA)/DT400 system. (b) Larger aggregate (LA)/DT400 system.

parameter is used to quantify the change of free energy of the binary colloid-polymer thermodynamic interaction and is defined in terms of the energy of interaction between colloid-polymer, pure colloid, and pure polymer (Dickinson, 2003). In the phase separation of soy protein aggregate and dextran, the interaction through thermodynamic incompatibility cannot be excluded.

Compared Fig. 1a and b, we found that the DT400 concentration needed for phase separation decreased with increasing size of the aggregate and the stable region became narrower. For smaller aggregates with $R_h = 37.0$ nm, when the protein concentration was 1%, the DT400 concentration of phase separation was above 2%, but for larger aggregates with $R_h = 144.9$ nm, DT400 concentration needed for phase separation was only approximately 1.1%. This was also approved by Croguennoc et al. (2001a) that larger aggregates phase separate preferentially and at a given polysaccharide concentration, only aggregates larger than a particular size phase separate and this critical size decreases with increasing polysaccharide concentration.

Other difference between both mixtures is the threshold of phase separation. The total biopolymer concentration of SA/DT400 was about 3%, but the phase separation of LA/DT400 initiated at the biopolymer concentration of lower than 2%. Explanation is that excluded volume determines space occupancy in biopolymer solutions and the phase separation threshold. There is more overlapped space between two larger approaching aggregates, which increased the depletion effect. Depletion-flocculation is due to the difference in osmotic pressure between the solvent and the space between the particles from which the polymer has been excluded (Bourriot, Garnier, & Doublier, 1999b). When two colloidal particles approach each other such that their depletion layers (each colloidal particle is surrounded by a layer in which the centre of the polymer cannot penetrate, and this excluded layer is called the depletion layer) start to overlap. The polymer concentration in the overlap region is lower than the environmental concentration, which results in the osmotic pressure. Two colloidal particles will associate with each other due to the force which is given by the product of the osmotic pressure and the overlap volume. These factors are related to, respectively, the polysaccharide concentration and the sizes

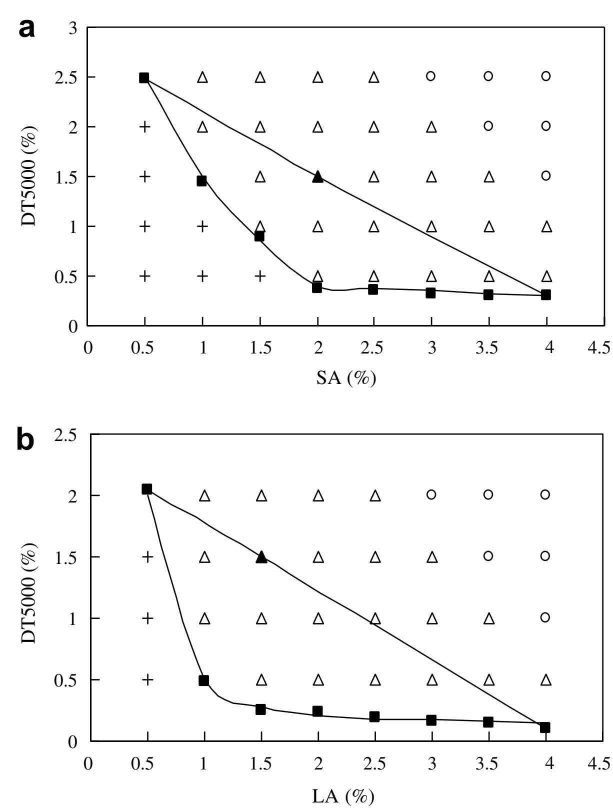

Fig. 2. Phase diagrams of soy protein aggregate/DT5000 system established at room temperature, pH 7.0. The solid line is the binodal line of phase separation; ▲, representative initial mixtures of soy protein aggregate/DT5000 for chemical analysis; ■, tie points (represent the compositions at upper and bottom phases separated from the initial mixtures, which obtained by chemical assays); +, stable mixtures; △, unstable mixtures; ○, gel points. (a) SA/DT5000 system. (b) LA/DT5000 system.

of the colloid and the polysaccharide (McClements, 2000). So in the system of soy protein aggregate and dextran, depletion interaction is also the possible reason for the phase separation.

3.1.2. Phase diagram of soy protein aggregate/DT5000 system

The phase diagrams for soy protein aggregate/DT5000 mixtures (Fig. 2) are similar to those of soy protein aggregate/DT400 mixtures; however, there are some different points.

(i) The stable regions were narrower than those of soy protein aggregate/DT400 mixtures, suggesting that the compatibility of DT5000 with soy protein aggregates decreased. This observation indicated that the dextran of high molecular weight accelerated the phase separation. Vrij (1976) demonstrated that the osmotic effect increases with increasing molecular weight and concentration of the added polymer. Quite small concentrations (order of per cents) should be effective with high molecular weight polymers. Li-In-On, Vincent, and Waite (1975) found that aqueous latex dispersions, sterically stabilized by poly(ethylene oxide) (PEO) chains, did flocculate above a certain, critical concentration of added PEO. The critical concentrations decreased from 55% to 27% with increasing molecular weight of the PEO ($M_w = 200$–4000).

(ii) The gelation areas were larger than those of soy protein aggregate/DT400 systems. From Doublier et al. (2000), when the polysaccharide was non-gelling, the resulting system was a gel which could appear homogeneous at the macroscopic level, though heterogeneous at the microscopic one. This explained the difficulties which were often encountered in the

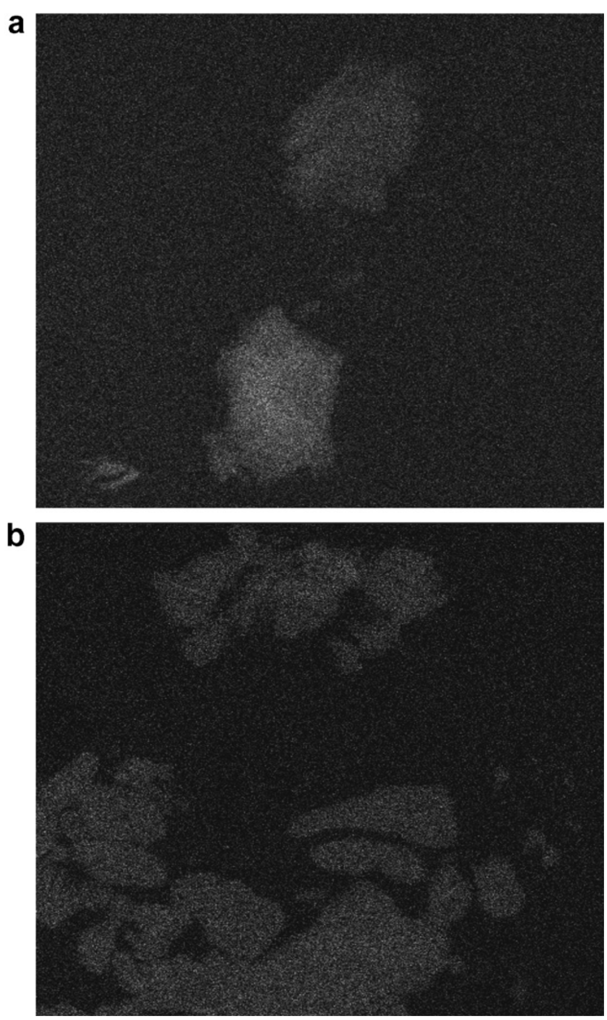

Fig. 3. CLSM images of mixtures containing 4% soy protein and 1% DT400. White color, protein-rich area; black color, polysaccharide-rich area. (a) SA/DT400 system. (b) LA/DT400 system. The bars are all 20 μm.

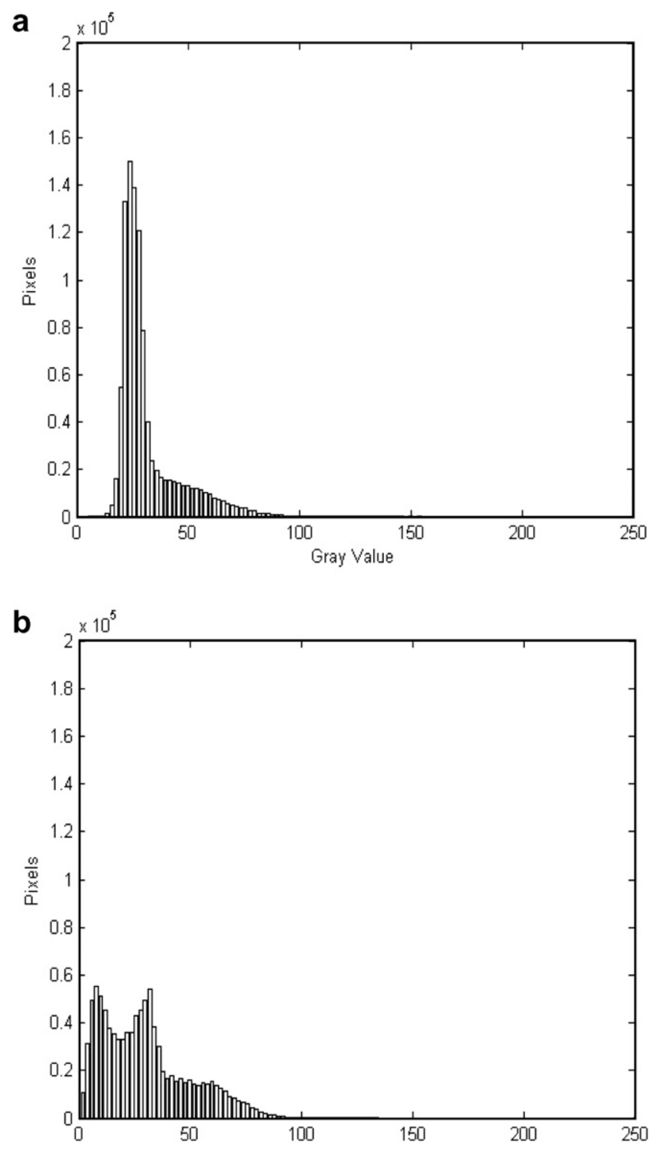

Fig. 4. Histograms of gray values for the mixtures of soy protein aggregates and DT400. (a) SA/DT400 system. (b) LA/DT400 system.

description of segregative phase separation. Our results suggested that gelation completed with phase separation simultaneously. The larger the size of the soy protein aggregate and/or the higher the molecular weight of the dextran were, the lower concentration of the polysaccharide was needed for forming the gel network.

3.2. Microscopic observations (CLSM) and image analysis

CLSM images of soy protein aggregate/DT400 systems are presented in Fig. 3 for mixtures consisting of 4% protein and 1% polysaccharide. Whatever the particle size of proteins in the mixtures (SA and LA), clear areas, corresponding to the fluorescence of FITC and thus revealing the presence of protein, were easily seen in the pictures. By contrast, dark areas corresponded to the localization of polysaccharide. It was thus seen that two macromolecular components of both mixtures were distributed into two separated phases. It also confirmed the results of phase diagrams that bulk phase separation took place in both systems.

These pictures also revealed the association of protein components. The volume of the protein-rich areas appeared to depend on the particle size of protein. In Fig. 3a, proteins appeared to concentrate in small volumes. DT400 constituted the continuous phase of the system. When the protein size was larger (LA, Fig. 3b), the volume of the fluorescent areas increased.

Based on the CLSM images (Fig. 3), we obtained the histograms of gray values (Fig. 4). In the all gray value range of the mixture of SA/DT400, only one narrow peak existed at the gray value of 25, which was the region of protein association; while in the system of LA/DT400, two peaks appeared, and located at about the gray value of

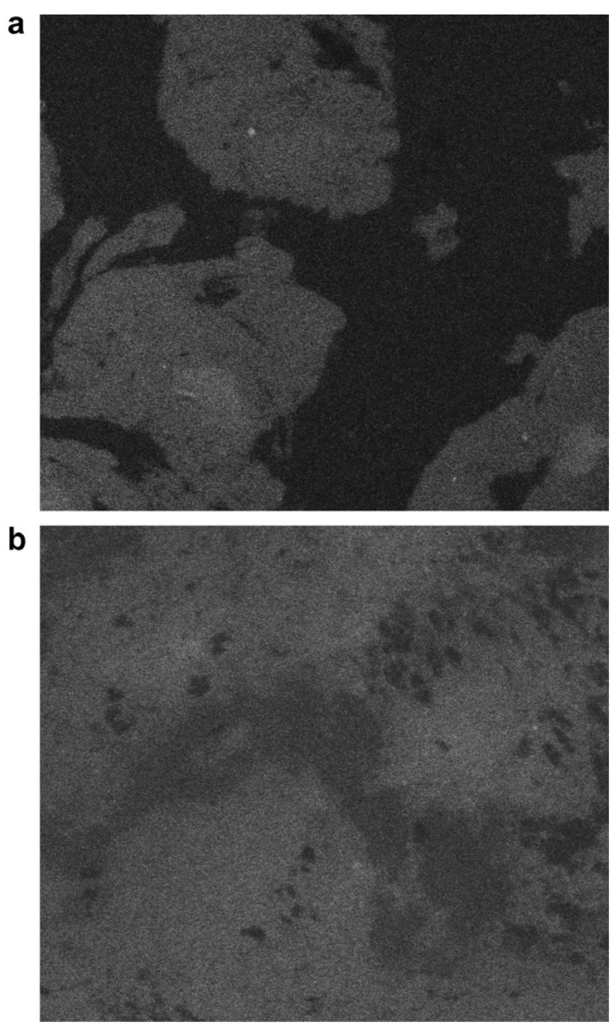

Fig. 5. CLSM images of mixtures containing 4% soy protein and 1% DT5000. White color, protein-rich area; black color, polysaccharide-rich area. (a) SA/DT5000 system. (b) LA/DT5000 system. The bars are all 20 μm.

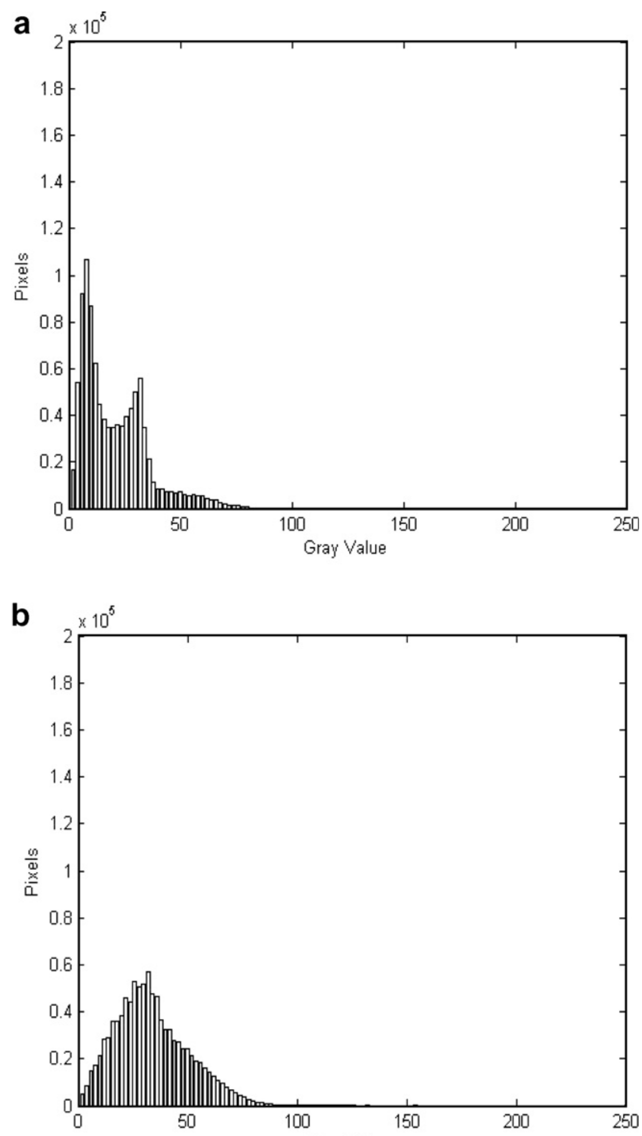

Fig. 6. Histograms of gray values for the mixtures of soy protein aggregates and DT5000. (a) SA/DT5000 system. (b) LA/DT5000 system.

10 and 30, respectively. One possible explanation for the latter is that protein association region distributes heterogeneously, i.e., distributes irregularly in the mixture, and a larger protein-rich region accompanies with polysaccharide-rich region.

In Fig. 5a (SA/DT5000), volume of protein-enriched areas increased markedly; two peaks appeared in its histogram of gray values (Fig. 6a) and the frequencies of both gray values increased much, which demonstrated that the protein associated parts were much denser. For the mixture of LA and DT5000, areas concentrated in protein were spread all over the picture; soy protein constituted the continuous phase of the system and performing a scan into the *z*-direction (*x*–*y* is the horizontal observation plane) of the LA/DT5000 system (up to 40 μm, de Bont, van Kempen, & Vreeker, 2002) revealed the interconnection of the association protein areas (network formation), and polysaccharides were trapped within the network. Corresponding to

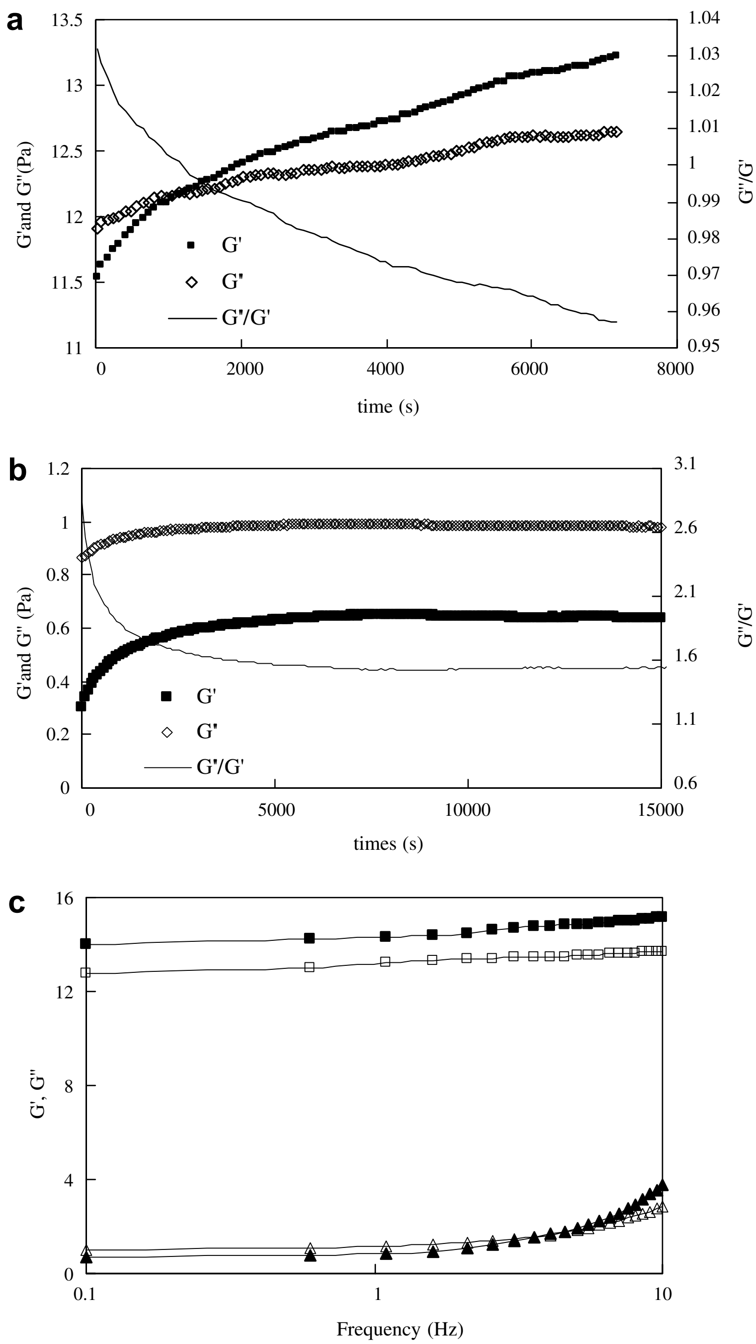

Fig. 7. (a) Variation of dynamic moduli (G′, G″) and tan δ (= G″/G′) as a function of time for LA/DT5000 system containing 4% protein and 1% polysaccharide. (b) Variation of dynamic moduli and δ as a function of time for SA/DT400 system containing 4% protein and 1% polysaccharide. (c) Variation of dynamic moduli as a function of frequency for systems after time sweep, ■ and ▲ represent LA/DT5000 and SA/DT400, respectively. Solid and open symbols represent G′ and G″.

the Fig. 2b, from a macroscopic point of view, a gel formed. One broad peak with larger gray value appeared in its histogram of gray values, which indicated that the degree of protein association were much larger. Above results suggested that the molecular weight of dextran and the size of aggregate affected the microstructures of phase separated systems.

We also found that during the initial stage of the phase separation, the bottom phases dissolved when diluted. This led to a one phase system, indicating that the process was reversible which was consistent with the depletion-flocculation mechanism (Blijdenstein, van Winden, van Vliet, van der Linden, & van Aken, 2004). However, the dissolution took more time the longer we waited before diluting the bottom phase. In addition, the bottom phase did not fuse as might be expected if they were truly liquid. These observations imply that the proteins in the bottom phase associate through physical bonds that are weak initially but which become stronger with time. The strengthening of the bonds occurs probably by restructuring so that, with time, more and more proteins are involved in the bonding. Possibly, hydrogen bonding or electrostatic interactions are involved.

3.3. Dynamic moduli of phase separated soy protein aggregate/dextran systems

Soy protein aggregate/dextran mixtures were characterized by small deformation measurements. Dynamic moduli (G', G'') were measured as a function of time during the phase separation process. Fig. 7a shows results for a mixture containing 4% LA and 1% DT5000. Initially, G' and G'' were small quantities with $G' < G''$. Both moduli gradually increased with time, indicating changes in the microstructure of the mixture. After about 1000 s, G' became larger than G'' ($\tan\delta < 1$). $G' > G''$ corresponds to the build up of a protein network-like structure in the soy protein/dextran system during phase separation. This is consistent with the observation of interconnected protein association structures in the confocal micrograph of phase separated mixture (Fig. 5b) and the model system containing latex colloids and hydroxyethylcellulose suggested by Sperry (1984). Ould Eleya and Turgeon (2000) also confirmed this protein network in the system of κ-carrageenan and β-lactoglobulin at the temperature heating to 90 °C. In the SA/DT400 system (Fig. 7b), G' and G'' ($G' < G''$) gradually increased with time, indicating some changes in the microstructure of the mixture, but after 4 h, G' was still smaller than G'', and both moduli became approximately steady. There were similar results in the other two systems. CLSM images also verified that in these mixtures, protein or polysaccharide-enriched regions distributed isolated.

We used another way to characterize the state of the systems which employs both G' and G'' and their frequency dependence (frequency sweep). Winter and Chambon (1986) found that for a typical gel, curves of G' and G'' are parallel to each other with $G' > G''$. In Fig. 7c, the G' and G'' curves for the mixture containing 4% LA and 1% DT5000 were parallel to each other and essentially independent of the oscillation frequency in the range of 0.1–10 Hz, indicating the formation of gel structure after time sweep. Those of SA/DT400 system crossed over, and demonstrated that the system did not transform to a gel.

4. Conclusion

The phase separation of soy protein aggregate/dextran mixture was studied. The results showed that molecular weight of dextran and size of soy protein aggregate affected the phase behavior of the mixtures, i.e., shifted the boundary of the instability region and the gelation areas. CLSM observations demonstrated an effective and inhomogeneous association between soy protein aggregates. Dynamic moduli measurements provided some additional information of microstructures. Our experiments showed that in soy protein/dextran mixtures at pH 7.0, phase separation through thermodynamic incompatibility cannot be ruled out, and depletion-flocculation is also the most likely cause of phase separation. The pure soy protein system after heat-treatment was expected to show similar phase behavior to the mixed system. The similarities of the microstructures will be studied further by optical microscopy and rheological measurement.

Acknowledgements

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