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Short communication: Changes in fluorescence intensity induced by soybean soluble polysaccharide–milk protein interactions during acidification

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ABSTRACT

Interactions between stabilizer and milk protein are believed to influence the stabilizing behavior of the milk system. We investigated changes in fluorescence intensity induced by interactions of soybean soluble polysaccharide (SSPS) and milk protein (Mp) during acidification. The fluorescence intensity (I_f) of Mp increased as pH decreased from 6.8 to 5.2. Compared with Mp alone, I_f of SSPS-Mp mixtures increased as the pH decreased from 6.8 to 5.2. We found that the I_f of the SSPS-Mp mixture decreased in a pH range from 5.2 to 3.6, which indicated a change in the polarity microenvironment around the Trp residues. We also found that the maximum emission wavelength (λ_{max}) shifted from 337 to 330 nm as pH decreased from 6.8 to 3.6, in further support of SSPS interacting with the polar portion of Mp during acidification. Furthermore, an excited monomeric molecule (pyrene exciplex) was found as a ground-state pyrene formed and a broad band was shown at about 450 nm. The intensity ratio of the first peak to the third peak (I_1:I_3) of Mp increased slightly, and the ratio of intensity of pyrene exciplex to monomer (I_e:I_m) decreased because pyrene molecules were located in a less hydrophobic microenvironment during acidification. However, the ratio of I_1:I_3 decreased clearly at pH below 5.6 and the ratio of I_e:I_m showed the opposite trend in the SSPS-Mp mixture. Changes in intrinsic and exogenous fluorescence intensity confirmed that interactions of SSPS and Mp could change the polarity of the microenvironment and that SSPS probably interacted with the polar portion of Mp. These results could give insight into the behavior of stabilizers in acid milk products.

Key words: soybean soluble polysaccharide, milk protein, fluorescence intensity, acidification

Short Communication

Caseins are the main proteins in milk and they play a major role in imparting structure to dairy products. In general, caseins are solubilized in the form of casein micelles under neutral pH conditions and they precipitate or aggregate under acidic pH conditions. The pH of acidified milk drinks ranges from 3.4 to 4.6, and because of the instability of caseins in this pH range, a stabilizer needs to be added to prevent protein aggregation and achieve optimal mouthfeel (Nakamura et al., 2006; Corredig et al., 2011).

Protein–polysaccharide interactions are important in the formulation of acidified milk drinks. Soybean soluble polysaccharide (**SSPS**) extracted from soybean cotyledons is often used to stabilize acidic beverages, which could improve the stability and texture of acidified milk drinks under low pH (acidic) conditions (Pereyra et al., 1997). Soybean soluble polysaccharide is negatively charged and fully adsorbs to the surface of positively charged proteins in acidic beverages. The steric repulsive force of SSPS adsorbed on the surface of casein explains stability at low pH, and the electrostatic repulsive force of polysaccharide chains contributes to the ability of SSPS to disperse milk proteins (Nakamura et al., 2003; Nobuhara et al., 2014).

The fluorescence in milk allows monitoring of structural modifications of proteins and their physicochemical environment during the coagulation process. Herbert et al. (1999) showed that a fluorescence method allowed detection of structural changes in milk during coagulation and discrimination of different dynamics of the coagulation system. In milk, most of the intrinsic fluorescence intensity can be attributed to the caseins present in the casein micelles (α_{S1}-, α_{S2}-, β-, and κ-caseins) and whey proteins (α-LA, β-LG, BSA; Fox, 1989; Tayeh et al., 2009; Rahimi and Corredig, 2012). The fluorescence of protein usually comes from the residues of Phe, Tyr, and Trp; Trp fluorescence is widely used to study interactions between protein and other molecules because of the high sensitivity to the polarity of its environment (Liu and Guo, 2008a,b). Be-

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cause α_S-casein and BSA have 2 Trp residues, β-casein and κ-casein have 1 Trp residue, and α-LA has 4 Trp residues (Kronman et al., 1972; Fox, 1989; Vanhooren et al., 2006; Tayeh et al., 2009; Rahimi and Corredig, 2012), structural alterations of the proteins could be detected by Trp fluorescence parameters. It could provide important information about casein micelles as well as the exogenous probe (Chakraborty and Basak, 2007).

In this study, we measured the fluorescence intensity of milk protein (**Mp**) alone and SSPS-Mp complex as a function of pH value. We aimed to study the interaction of Mp and SSPS during acidification and to evaluate the kinetics of coagulation and structural aspects of the stabilizing layer. Our results could have important theoretical and practical implications in the stabilization of acid milk products.

Skim milk powder was obtained from Fonterra Cooperative Group (Wellington, New Zealand). The SSPS was donated by Fuji Oil Co. Ltd. (Osaka, Japan) and was prepared from the residue of protein extraction, as previously described (Nakamura et al., 2001, 2006). Pyrene was purchased from Fluka Company (Buchs, Switzerland), and citric acid was obtained from Zhanwang Chemical Co. Ltd. (Wuxi, China).

The reconstituted milk with a content of 2% (wt/vol) protein was prepared by mixing skim milk powder with milli-Q water (Millipore Corporation, Billerica, MA). Meanwhile, SSPS was prepared in deionized water, stirred at 70°C for 30 min, and stored at room temperature to allow complete hydration. The final concentration in mixtures was calculated to obtain samples with 1% (wt/vol) proteins and 0.5% (wt/vol) SSPS; the mixtures were made from the reconstituted milk and SSPS hydrates.

Determination of Trp fluorescence was performed by using an F-7000 type spectrometer (Hitachi, Tokyo, Japan). The SSPS-Mp mixtures prepared with different pH values were diluted 10 times before determination of Trp fluorescence. The excitation and emission slits were fixed at 3.0 and 1.5 nm, respectively. The excitation wavelength was set at 297 nm and the emission spectra were collected from 290 to 400 nm. The voltage was set at 400 V. Pyrene was used as a neutral hydrophobic probe to determine the microenvironment polarity of Mp. The emission spectra were measured at the range of 300 to 600 nm, and the excitation wavelength was 335 nm.

Experimental data were analyzed using ANOVA, and data were expressed as mean values ± standard deviations. Duncan's multiple range test was performed for post hoc multiple comparisons with the level of significance set at $P < 0.05$. All statistical analyses were performed using SPSS 18.0 software (SPSS Inc., Chicago, IL).

The fluorescence spectra of SSPS-Mp mixtures under different pH conditions are shown in Figure 1. The fluorescence intensity ($\mathbf{I_f}$) of Mp without addition of SSPS increased as pH decreased from 6.8 to 5.2, which can be explained by the loose structure of casein micelles released during acidification and exposure of Trp residues. Acidification caused the loss of casein micelles, which presumably originated from proteins that had dissociated from the casein supramolecules (McMahon et al., 2009). Compared with Mp alone, I_f of SSPS-Mp mixtures increased significantly as the pH decreased from 6.8 to 5.2 ($P < 0.05$). This result was probably consistent with the turbidity based on the inner filter effect of casein, whereby excitation or emission of light absorption led to a reduction in fluorescence intensity (Klockenkamper, 1997). At pH <5.2, casein in the Mp samples precipitated and no fluorescence experiment was conducted. Meanwhile, no fluorescence emission was found for SSPS (the blank sample without Mp). In the SSPS-Mp mixtures, values of I_f decreased as pH decreased from 5.2 to 3.6, which indicated variation in the polarity microenvironment around the Trp residues as well as the inner filter effect.

We found that the maximum emission wavelength ($\boldsymbol{\lambda_{max}}$) shifted from 337 to 330 nm as pH decreased from 6.8 to 3.6 (Figure 1B). The λ_{max} of Trp residues shifts to shorter wavelengths when Trp residues move to a less polar microenvironment (Liu and Guo, 2008a,b). This finding could indicate that SSPS probably interacted with the polar portion of Mp, whereas the nonpolar portion formed a more hydrophobic domain where Trp residues were located, resulting in a continuous decrease of λ_{max} during acidification.

The influence of pH on SSPS-Mp mixtures was also detected by the pyrene probe measurement (Figure 2). The intensity ratio of the first peak to the third peak ($\mathbf{I_1{:}I_3}$) of the fluorescence spectrum of pyrene indicated the polarity of the environment. The lower value of $I_1{:}I_3$, the greater hydrophobicity the system had (Keyes-Baig et al., 2004). An excited monomeric molecule (pyrene exciplex) was found as a ground-state pyrene form, which was observed as a broad band at about 450 nm. The ratio of intensity of pyrene exciplex to monomer ($\mathbf{I_e{:}I_m}$) was used to provide information about hydrophobic domains in the system (Sahoo et al., 2000). Because the sample of Mp alone precipitated at pH <5.2, no fluorescence experiment was conducted. The data were obtained from pH 6.8 to 5.2. The ratio of $I_1{:}I_3$ increased slightly (from 1.001 to 1.018) and the ratio of $I_e{:}I_m$ decreased (from 0.282 to 0.156) with decreasing pH, which indicated that pyrene molecules were located in a less hydrophobic microenvironment because of the loose structure of casein micelles during acidification.