Phosphorus-31 Nuclear Magnetic Resonance Study on Adsorption Behavior of Caseinate in Triacylglycerol-in-Water Emulsions

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^{31}P NMR was used to study the adsorption behavior of caseinate in triacylglycerol-in-water emulsions. The droplet size and surface areas of caseinate-stabilized emulsion are closely related to the oil/protein ratio in emulsions. The line widths of ^{31}P NMR spectra of the protein in emulsion increased with increase of the amount of protein adsorbed at an oil-in-water interface. Thus, the restriction of the phosphoserine residues of caseinate was correlated with the interaction between protein and triacylglycerols on the interface. The droplet size of the emulsion became larger with increasing carbon number of saturated triacylglycerols but became smaller when unsaturated triacylglycerol was used as an oil phase. Broadening of ^{31}P NMR spectra also suggested the possibility of a conformational change of caseinate induced by varying the temperature and molecular species of triacylglycerols at the interface.

Keywords: *Caseinate; triacylglycerol; emulsion; adsorption behavior; ^{31}P NMR; relaxation time*

INTRODUCTION

Emulsification properties is one of the most important functional properties of food proteins, and many studies have been made by many researchers (Dickinson *et al*., 1988; Das and Kinsella, 1990; Ericsson, 1990; Mangino, 1994; Meste and Davidou, 1995). Hydrophobicity (Nakai, 1983) and flexibility (Kato and Yutani, 1988) are generally accepted to be the major factors which decide emulsifying properties of proteins. However, adsorption behavior and conformation of the adsorbed proteins at the interface are little understood. Elucidation of the structure of proteins at the interface is essential to understand the mechanism for emulsification and the stabilization of emulsion by proteins.

Casein and caseinate are well known to be good protein emulsifiers (Mulvihill and Fox, 1989). The structure of protein adsorbed to an oil-in-water and solid−water interfaces has been studied by means of proteinase (Shimizu *et al*., 1986; Dalgleish and Leaver, 1991; Leaver and Dalgleish, 1992a,b; Saito *et al*., 1993), light scattering (Morrissey and Han, 1978), CD (Kondo *et al*., 1991; Norde and Favier, 1992; Smith and Clark, 1992), DSC (Haynes and Norde, 1995), and FT-IR (Jakobsen and Wasacz, 1987) spectroscopic techniques. Among these techniques, proteases such as trypsin and α-chymotrypsin are widely used for this purpose. Shimizu *et al*. (1986) showed that some hydrophobic regions of an αs_1-casein chain might interact strongly with oil, while the other hydrophilic regions might not. The charged N-terminal region of β-casein in a soya oil−water interface is more accessible to trypsin-catalyzed hydrolysis (Leaver and Dalgleish, 1992a,b).

The phosphorus NMR technique has been applied in elucidating the interfacial adsorptivity and dynamic structures of phospholipids (Chiba and Tada, 1989, 1990) and phosphoproteins (Mine *et al*., 1992a) or phospholipid−protein interactions (Mine *et al*., 1992b,c, 1993) at an oil-in-water interface by observing the motional property of phosphoserine of phospholipids and phosphoproteins. This technique provides useful information in lipid−protein interaction at an oil-in-water interface *in situ*. Casein protein is one of the well-known phosphoproteins. The phosphate is esterified to the polypeptides as monoesters of serine (rarely of threonine) (Fox, 1989). αs_1-Casein usually contains 8 mol of P/mol of protein and β-casein usually contains 5 mol of P/mol of protein. αs_1-Casein is also the first phosphoprotein to be studied by ^{31}P NMR (Ho and Kurland, 1966). Thus, the ^{31}P NMR technique can be expected to be applied in evaluating the emulsifying properties of caseinate *in situ*.

In this work, high-resolution ^{31}P NMR was used to investigate the adsorption behavior of caseinate in triacylglycerol-in-water emulsions. The relationship between the molecular structure of triacylglycerol and adsorptivity of caseinate proteins was also discussed.

MATERIALS AND METHODS

Materials. Caseinate, trioctanoylglycerol (tri-8:0), tridecanoylglycerol (tri-10:0), trilauroylglycerol (tri-12:0), and trioleolylglycerol (tri-18:1) were purchased from Sigma Chemical Co., St. Louis, MO. Caseinate was dissolved in water and precipitated by acidifying to pH 4.5. This was dissolved by adjusting the pH to 7.5 and precipitating the casein with acid again. The precipitate was redissolved at pH 7.0, dialyzed against distilled water, centrifuged, and freeze-dried. This treatment was effective to remove the salts such as phosphorus compounds in the sample. Triacylglycerols were purified by silica gel column chromatography (hexane−diethyl ether, 97:3) until more than 95%, as shown by gas liquid chromatography analysis.

Emulsion Preparation. The caseinate solution (0.5% in 20 mM imidazole buffer, pH 6.0, containing 10 mM EDTA) was mixed with various amounts of triacylglycerols. Oil-in-water emulsions were prepared using a high-speed homogenizer (Cole Parmer Instrument Co., Chicago, IL) with a generator shaft (10 mm diameter) at 12 000 rpm for 1 min and further homogenized using an ultrasonic homogenizer (Model 470, Cole Parmer Instrument Co., Chicago, IL) at 40 W output power for 2 min.

Particle Size Analysis. The droplet size distribution and the surface area of the oil in each emulsion were measured by a laser diffraction particle analyzer (Model SALD-2000, Shimadzu Corp., Kyoto, Japan). The surface measured by the analyzer was used for the calculation of the surface coverage of adsorbed caseinate. The refraction index used here was

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1.60. The data are expressed as a mean volume surface diameter ($d_{3,2}$).

Determination of Surface Concentration of Protein. Emulsions which were composed of 0.5% protein solution (4 mL) and various amounts of triacylglycerols were centrifuged at 15000*g* for 1 h according to a previous paper (Mine *et al.*, 1992a). The water layer was withdrawn. The cream phase was then resuspended in buffer so that the weight fraction of the two phases was as in the original emulsion. The sample was centrifuged in the same way, and the water layer was withdrawn. The first and second water layer samples were combined and analyzed for protein by the Lowry method (Lowry *et al.*, 1951). The total amount of protein adsorbed was measured by subtracting the protein amount in the water phase from the total added protein after separating the water and oil phases by the above method. The cream phase was subjected to SDS−polyacrylamide gel electrophoresis after extracting the adsorbed protein by 8 M urea and 5% 2-mercaptoethanol in boiling water.

SDS−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−polyacrylamide gel electrophoresis was done by the standard method of Laemmli (1970). The electrophoresis was done using a minislab gel (72 × 90 × 1.0 mm, 4−20% in the gradient-separating gel and 3% in the stacking gel). Gels were stained with Coomassie brilliant blue R-250.

^{31}P NMR Measurement. ^{31}P NMR measurements were performed on a Varian VXR-4000s spectrometer at 161.0 MHz fitted with a probe (10 mm, 45−165-MHz frequency), using a 45° pulse (25 μs), with 32K data points, a 40000-Hz spectral window, 20 rotations/s spinning rate, and 2.0-s pulse delay. Each proton was fully decoupled by a 9900-Hz decoupling modulation frequency, and the NMR samples were 3.1 mL in 10-mm precision tubes. The line widths were measured from the resonances at half-height. The relaxation parameters described below do correlate with structural features of molecules and particularly with their motions, and the relaxation measurements are sometimes used to good effect in structure elucidation. Spin−lattice relaxation time (T_1) was measured by an inversion recovery (180°−τ−90°) pulse sequence with a 90° pulse width of 50 μs; 2000 acquisition times were accumulated for each 6-s delay time τ. Spin−spin relaxation time (T_2) was measured by the spin-echo method with a 90°−τ−180° pulse sequence (Derome, 1987); 2000 acquisition times were calculated by a computer performing a nonlinear regression of the exponential to the corresponding curves. The samples contained 20% ^2H_2O for internal locking.

RESULTS AND DISCUSSION

Figure 1 shows the mean droplet size and surface area for caseinate-stabilized emulsions as a function of oil content. The particle size increased with increasing oil content from 0.1 to 1.0 g in 4 mL of 0.5% protein solution. Particle size of the emulsion was 0.43 μm at 0.1 g of oil content, and it was increased to 1.68 μm at 1.0 g of oil content (Figure 1a). Figure 1b shows the specific surface area derived from the particle size distribution plotted against oil content of emulsion. The specific surface area formed by caseinate decreased linearly with increasing oil content as a result of formation of larger droplet size of emulsions. These data indicate that the droplet size and surface areas of caseinate-stabilized emulsions are closely related to the ratio of oil/protein in the emulsion.

Figure 2 shows typical ^{31}P NMR spectra of caseinate in a solution and in emulsions at the various oil contents. A single spectra with a 19.6-Hz line width for caseinate in solution was observed (Figure 2a). This signal must have originated from phosphoserine in β-casein and αs_1-casein. However, most of previous reports of casein phosphoserine signals have found a number of peaks spread over a range of several parts per million (Humphrey and Jolley, 1982; Sleigh *et al.*, 1983; Wahlgren *et al.*, 1986; Belton and Lyster, 1985, 1991). Interestingly, most of these signals observed in an early NMR study of milk have been assigned to phosphorus compounds already known to be present in milk or casein samples (Wahlgren *et al.*, 1986; Belton and Lyster, 1991). Therefore, it is very important to remove these phosphorus compounds in samples before use to avoid the detection of such impurities by ^{31}P

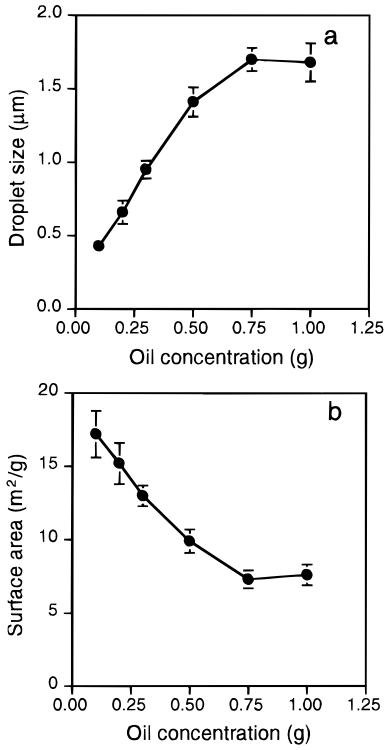

Figure 1. Mean diameter of oil droplet and surface area as a function of tri-18:1 concentration. Median diameter of droplet and surface area were derived from measuring the droplet size distribution using a laser diffraction particle analyzer: (a) change in oil droplet size and (b) change in surface area. Data are the average of triplicate measurements.

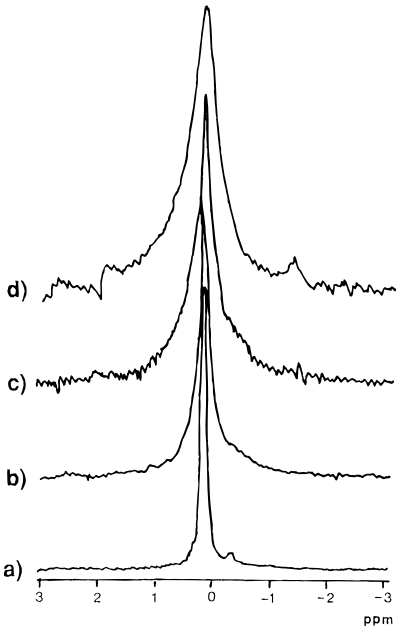

Figure 2. ^{31}P NMR spectra of caseinate in an aqueous dispersion and emulsions: (a) the solution of 0.5% caseinate and (b−d) emulsions. The tri-18:1 contents were 0.2 g (b), 0.5 g (c), and 1.0 g (d) in 4.0 mL in 0.5% protein solution, respectively.

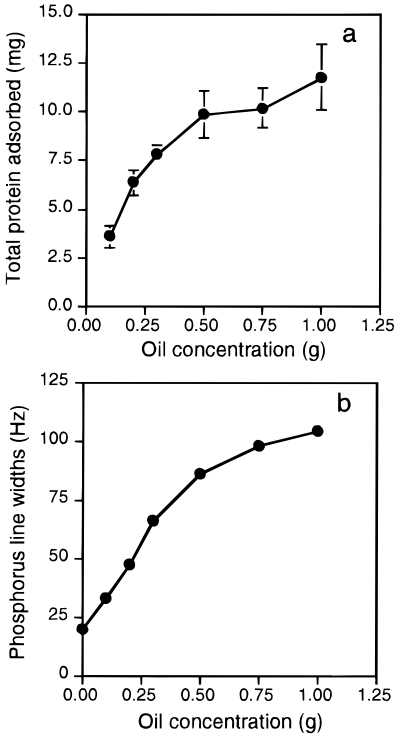

Figure 3. Changes of total amounts of adsorbed proteins (a) and phosphorus line widths (b) of caseinate-stabilized emulsions as a function of tri-18:1 concentration. Data are the average of triplicate measurements.

NMR. Sleigh *et al.* (1983) observed two and four spread multiple resonances of β-casein and αs_1-casein at pH 6.8 using high-resolution ^{31}P NMR, respectively. They also reported the pH-dependent change in the chemical shift of the ^{31}P resonance of phosphoserine in αs_1-casein. This is also observed in the case of β-casein phosphoserine by Humphrey and Jolley (1982). However, these signals are still not assigned in detail. In this light, the resonance of ^{31}P NMR spectra of caseinate must be affected by pH. In the present work, the minor peaks, which should be observed at alkaline pHs, can be hidden by the major one because the pK_2 of the downfield phosphate resonance of casein is 5.9 and that of the upfield phosphate resonance is 6.65. Thus, it is suggested that there are no differences between the environments of individual phosphoserine residues of the caseinates at pH 6.0. In the emulsion of caseinate, the phosphorus line widths became broader with the increase of oil content (Figure 2b,d). The phosphorus signals of ^{31}P NMR spectra are influenced by the motional properties of the phosphate moiety in the molecules, and peak broadening of phosphorus signals of ^{31}P NMR spectra is observed by increasing magnitude of the negative phosphorus chemical anisotropy (Wu *et al.*, 1984). The peak broadening of ^{31}P NMR spectra with the increase of oil content in emulsion is considered as the decrease of motions of the phophoserine moiety in caseinate as a result of adsorbing caseinate on the interface. These data indicate that the extensive peak broadening of ^{31}P NMR spectra of caseinate in emulsion is correlated with the adsorption behavior of protein adsorbed at the oil-in-water interface.

Figure 3 shows the relationship between the line widths of ^{31}P NMR spectra of caseinate and its total amount of protein adsorbed at the interface as a function of oil content. The total adsorbed protein was increased with increase of oil content. The amount of the protein was in excess when the oil content was 0.1 g, and 80% of the protein remained in serum. The excess protein existing in a serum was adsorbed on the interface with increasing oil content, and the total amount of adsorbed protein reached was 64% at the oil content of 1.0 g. The line width of ^{31}P NMR spectra was increased from 19 to 101 Hz in relation to an increase of oil content, and the peak broadening of the signals was well correlated to the amount of adsorbed protein. These data are interpreted as indicating that the motional freedom of the phosphoserine moiety in caseinate was restricted due to the increase of adsorbed proteins at an oil-in-water interface. The phosphorylation groups of αs_1-casein are located between 41 and 81, which contain the eight phosphoseryl residues. It has three strongly hydrophobic regions: residues 1−40, 90−113, and 132−199 (Fox, 1989). It was reported that some hydrophobic regions of an αs_1-casein, the neighborhood of the 21−25, 94, 135, and 142 from the N-terminal, might interact strongly with oil (Shimizu *et al.*, 1986). The N-terminal section (residues 1−21) of β-casein is highly charged and contains four of five phosphoseryl residues (Fox, 1989). Leaver and Dalgleish (1992a,b) reported that the N-terminal peptides 1−25 and 1−28 of β-casein, a hydrophilic region, might protrude from the interface. Therefore, the peak broadening of ^{31}P NMR spectra of caseinate in emulsion indicates a slow motion of phosphate groups in the protein as a result that the hydrophobic region of αs_1-caseinate and β-caseinate, the neighborhood of the phosphorylated regions, might be adsorbed on the interface.

Table 1. T_1 and T_2 Relaxation Times of Caseinate in Solution and Emulsion

	T_1 (s)	T_2 (ms)
solution	1.42 ± 0.04	26 ± 4
emulsion	0.17 ± 0.02	8 ± 2

In order to ascertain the motional properties of phosphoserine in caseinate for an emulsion, the T_1 and T_2 relaxation times for caseinate in an aqueous dispersion and in an emulsion were measured (Table 1). The T_1 relaxation time of caseinate phosphate residues in emulsion was markedly decreased from 1.42 to 0.17 s. The T_2 relaxation time was also decreased from 26 to 8 ms in emulsion. In general, the relaxation time strongly depends on the environment and the mobility of the observed nuclei (Hart, 1984; Derome, 1987). Thus, the data obtained from the relaxation time measurements indicate not only the restriction of phosphoserine motional freedom derived from the adsorption of the protein at an oil-in-water interface but also the possibility of its conformational change at the interface. In other words, the peak broadening of ^{31}P NMR spectra and the relaxation time measurements in caseinate can be used as a sensitive probe in evaluating the adsorption behavior of caseinate at an oil-in-water interface.

Figure 4 shows the changes of phosphorus line widths of caseinate in solution and the emulsion containing 20% of oil as a function of temperature. In the protein solution, the line width scarcely changed despite the increase of temperatures. On the other hand, the line width gradually increased in the emulsion of caseinate with tri-18:1 when the temperature was elevated at 5 °C/min in the sample tube. The line width reached to about 1.6 time at 80 °C as compared to the one at 20 °C. The droplet size of caseinate emulsion was not changed with increasing temperature. It is suggested that the environments of individual phosphoserine residues varied with the temperature at the interface.

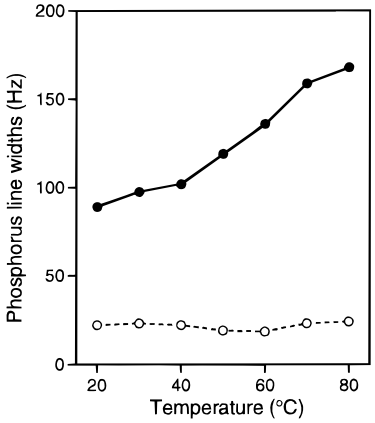

Figure 4. Phosphorus line widths of caseinate as a function of temperature: (○) in the solution and (●) in the emulsion composed of tri-18:1. The emulsion was composed of 0.5% protein solution (4 mL) and tri-18:0 (1.0 g) at 20 °C, and the temperature was raised in stages from 20 to 80 °C at 5 °C/min.

This general feature of the observations indicates that the affinity between caseinate and the oil phase was changed with the temperature or aggregation of caseinate molecules formed at the interface. It is possible that the increased affinity between the protein and oil phase or the formation of protein aggregates might cause the broadening of the line widths.

Elucidation of the relationship between the molecular structure of triacylglycerols and emulsifying properties of proteins is of great interest to clarify the lipid−protein interactions. However, most of studies on emulsifying properties have been focused on the properties of proteins. There was little information on the relationship between the molecular structure of triacylglycerols and adsorption behavior of proteins until the recent report of Kimura *et al*. (1994). As described previously, ^{31}P NMR technique provides useful information in lipid−caseinate interaction at an oil-in-water interface *in situ*. Consequently, an attempt was made to evaluate the adsorption behavior of caseinate at various triacylglycerol emulsions using this technique. Figure 5 shows the particle size distribution of caseinate-stabilized emulsions composed of tri-8:0, tri-10:0, tri-12:0, and tri-18:1. The preparation of the emulsions and measurements of the droplet size were done at 60 °C to avoid the crystallization of oils used herein. In the emulsion of tri-8:0, the protein formed smaller droplet sizes with a mean diameter of 5.25 μm and gave a homogeneous pattern in particle distribution. However, the droplet size of the emulsion became larger with increasing carbon number of saturated molecular species and showed more heterogeneous and spread patterns. On the other hand, the emulsion formed smaller droplets when tri-18:1 was used as an oil phase. These results indicate that the emulsifying properties of caseinate were affected by the carbon number and the double-bond number of triacylglycerols. It was also suggested that the adsorption behavior varied with the molecular structure of the oils.

To determine the differences of the protein adsorptivity with various triacylglycerols, the composition of adsorbed caseinate and the phosphorus signals of the protein in emulsion were analyzed (Figure 6 and Table 2). No preferential adsorption was observed among caseinate components which were adsorbed at the interface with the various triacylglycerols species (Figure 6). Broadening of phosphorus signals on tri-10:0

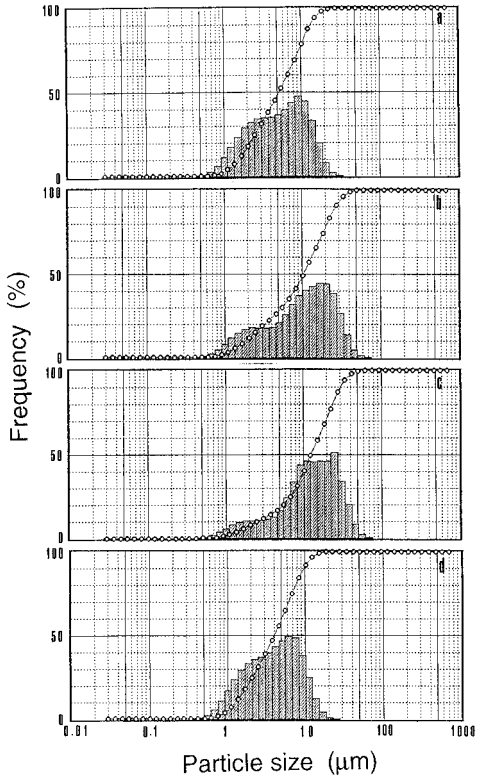

Figure 5. Particle size distributions of emulsions composed of caseinate and various triacylglycerols. The emulsions were composed of 0.5% protein solution (4 mL) and various triacylglycerols (1.0 g) and prepared at 60 °C. The droplet size was measured by the volumetric percentage: (a) tri-8:0, (b) tri-10:0, (c) tri-12:0, and (d) tri-18:1 emulsions.

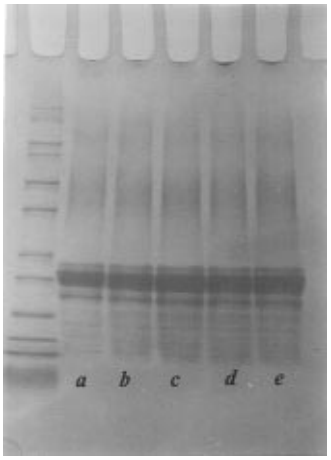

Figure 6. SDS−PAGE patterns of caseinate proteins adsorbed on various triacylglycerols emulsions. The conditions of emulsification were the same as in Figure 5: (a) control, (b) tri-8:0, (c) tri-10:0, (d) tri-12:0, and (e) tri-18:1 emulsions.

and tri-12:0 emulsions was observed, even though the total amount of adsorbed protein was the same as those of tri-8:0 and tri-18:1 emulsions. The surface concentration of the protein in each emulsion as a function of triacylglycerol species was significantly different among each emulsion. The surface concentration of the protein in tri-8:0 and tri-18:1 emulsions showed 3.91 and 3.07 mg/m^2, respectively. On the other hand, the surface concentration for tri-10:0 and tri-12:0 emulsions was increased to 1.9−2.3 times higher than that for the tri-18:1 emulsion. These data suggest that the caseinate might form a more condensed or thicker film at a long-carbon-saturated triacylglycerol-in-water interface than in short-carbon-saturated or long-carbon-unsaturated

Table 2. Relationship between Emulsification Properties and Phosphorus Signals of Caseinate as a Function of Triacylglycerol Molecular Species[a]

oil type	mean diameter (μm)	surface concentration (mg/m^2)	total adsorbed protein (mg)	^{31}P NMR (Hz)
trioctanoylglycerol	5.25 ± 0.36	3.91 ± 0.18	7.21 ± 0.12	101.9
tridecanoylglycerol	10.57 ± 0.43	5.65 ± 0.09	7.13 ± 0.14	149.8
trilauroylglycerol	12.72 ± 0.40	6.94 ± 0.22	6.89 ± 0.16	192.8
trioleolylglycerol	4.05 ± 0.33	3.07 ± 0.16	7.01 ± 0.19	94.7

[a] The preparation of the emulsions and measuring the droplet size and ^{31}P NMR spectrum were done at 60 °C. The emulsions composed of 0.5% protein solution (4 mL) and triacylglycerol (1.0 g) were prepared using a high-speed homogenizer at 12 000 rpm for 2 min. Data are the mean value of duplicate measurements.

ones. Broadening of ^{31}P NMR spectra in long-carbon-saturated triacylglycerol emulsions also suggests the possibility of a conformational change of caseinate proteins induced by varying the affinity between the protein and oil phase or forming protein aggregation at the interface. Another reason for these phenomena was that the motional freedom of the phosphoserine in caseinate was more restricted due to the changes of the interaction between the protein and the packing with triacylglycerol. Therefore, it is difficult to conclude this problem by only peak broadening of the ^{31}P NMR spectrum. The T_1 relaxation time and the nuclear Overhauser effect measurements are required to clarify the intra- and intermolecular interaction of the protein at an oil-in-water interface.

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