Effects of Fat Crystallization on the Behavior of Proteins and Lipids at Oil Droplet Surfaces

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ABSTRACT: The effects of fat crystallization induced by thermal treatment on the rheological properties of creams and physical phenomena at the oil droplet surfaces were investigated. Creams A or B were prepared from commercial proprietary fats A or B (vegetable oils with different triacylglycerol composition) and aqueous solution containing proteins. Thermal treatment of the creams at the "critical temperatures" (temperatures inducing a small percentage of solid fats in the oil droplets) caused a rapid increase of solid fat contents in the following cooling process. The thermal treatment of cream B at the "critical temperature" caused an increase of viscosity of the cream and an increase of protein surface coverage during the subsequent cooling process. These results suggest that the oil droplet aggregation induced by the thermal treatment at the "critical temperature" and the subsequent cooling occurred *via* further adsorption of proteins. Electron spin resonance measurement demonstrated the dramatic reduction of fluidity of triacylglycerol molecules at the oil droplet surface in cream B during the cooling process after thermal treatment at temperatures below "critical." Based on these results, we speculated on the mechanism for the destabilization of thermally treated creams during the cooling process.

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KEY WORDS: Cream, crystallization, ESR, fluidity, oil droplet surface, protein adsorption, pulsed NMR, solid fat contents, thermal treatment, viscosity.

Industry demands food creams with good qualities that are resistant to destabilizing factors, such as heating and vibration during transportation. The stability of cream against changes in storage temperature is especially of great importance (1). It is well-known that "heat-shock," i.e., a temporary elevation of storage temperature, leads to coagulation or an increase of the cream after the recooling process (2). Though the direct reason for the coagulation or viscosity increase remains unclear, it is likely that crystallization of fat in the oil droplets of the cream is responsible for such a phenomenon. This speculation is based on the previous observation that the degree of crystallization of fat during the thermal change affects the dispersion state of oil droplets as well as the distribution of emulsifiers between dispersed and continuous phases (3–5).

Many kinds of triacylglycerols are in the natural lipids used in commercial creams, and each triacylglycerol has a different melting point. Therefore, it is expected that the crystallization behavior of fats and the dispersion state of oil droplets in emulsions that contain natural lipids are more complicated than those of emulsions that contain a single kind of triacylglycerol.

Previously, Noda and Yamamoto (2) studied the stability against heat shock of emulsions prepared from vegetable oils, including various kinds of triacylglycerols with different melting points. They also analyzed the solid fat contents (SFC) in the oil droplets of emulsions by using a pulsed nuclear magnetic resonance (NMR) technique (6,7). The close relationship of emulsion stability with heat shock and SFC was demonstrated. That is, the thermal treatment at a temperature that generates SFC in the range from a small percentage to 10% in the oil droplets caused coagulation or a viscosity increase in the cream after a recooling process. The importance of partly crystalline droplets (SFC is a small percentage to 10%) with respect to the destabilization of emulsions, observed in References 2–5, agrees with earlier findings.

Thermodynamically, it is favorable for fat crystals to be located at the oil–water interface rather than in the interior of the oil droplets (2). Fat crystals therefore modify the physical state of oil droplet surfaces, such as the fluidity of triacylglycerol molecules, adsorption or arrangement of emulsifiers (such as proteins and low-molecular-weight surfactants), and zeta-potential, thereby influencing emulsion stability. There have been a few reports about the mechanism of heat-shock phenomena in relationship to partly crystalline droplets (2–5). In the present study, we investigated the changes of physical properties of emulsions as a function of temperature. The emulsions were prepared from the same vegetable oils used in a previous study (8), although the composition of emulsions was simpler. We analyzed rheological properties as well as the physical state of

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oil droplet surfaces, such as protein adsorption behavior and fluidity of triacylglycerol molecules.

MATERIALS AND METHODS

The bovine α-lactalbumin (type 1), α-casein, and 5- and 16-doxyl (4,4-dimethyloxazolidine-*N*-oxyl)-stearic acid (5-SA or 16-SA) were purchased from Sigma (St. Louis, MO). Sodium phosphate and other reagents of analytical grade were purchased from Nakarai Tesque (Kyoto, Japan).

Fat A and fat B (vegetable oils) were obtained from Snow Brand Milk Products Co., Ltd. The main components of the acyl chains of triacylglycerol in fat A and B are oleic and lauric acids, respectively.

Emulsion preparation. Oil-in-water creams were prepared from 40 wt% fat A or fat B and an aqueous solution that contained 2.0 wt% β-casein (10 mM sodium phosphate buffer, pH 7.0). The mixtures were homogenized for 3 min in a high-speed blender (Nichion Irikaki Seisakusyo Co. Ltd., Tokyo, Japan) at 22,000 rpm at 60°C. The diameter of the oil droplets was further reduced by ultrasonication in an ultrasonic homogenizer (Nihon Seiki Kaisha Co. Ltd., Tokyo, Japan) for 2 min at 60°C. The resulting emulsions were cooled rapidly and stored overnight at 5°C. A laser diffraction particle-size analyzer LA-500 (Horiba Seisakusyo Co. Ltd., Kyoto, Japan) was used to determine the droplet-size distribution of the emulsions, from which was derived the specific surface area (area per unit mass of emulsion).

Thermal treatment and cooling. Sample creams were incubated at various temperatures in the range of 25–55°C. After incubation at the set temperature for 1 h, creams were cooled to 5°C at the rate of 0.5°C/min. Creams were then incubated at 5°C for 1 h (Fig. 1). The various measurements that are described in the following sections were performed during this cooling process.

SFC measurement. SFC values of oil droplets of the creams were derived from pulsed NMR measurements (6,7), which were performed with a Minispec NMS-120 (Bruker Japan Co. Ltd., Tsukuba, Japan).

Rheological testing. Heat-treated creams were characterized in terms of their rheological properties. Small-deformation shear viscoelastic measurements (strain: 1.0%, frequency: 1.0 Hz) were made in a Rheosol-G3000 (UBM, Kyoto, Japan) with a cone and plate-type cell (cone angle: 1.97°, cone diameter: 39.98 mm).

Determination of adsorbed amount of protein at the oil-droplet surface. Creams were diluted 20 times by sodium phosphate buffer before thermal treatment. During the heating and cooling process, aliquots of creams were taken and centrifuged at 70,000 × *g* at various temperatures to separate the aqueous phase from the oil droplets. The aqueous phase was collected with a syringe and filtered through a membrane filter (pore size: 0.45 μm; Millipore, Bedford, MA). The amount of protein in the aqueous phase was determined by the method of Lowry *et al.* (9). The adsorbed amount of protein at the oil droplet surface (protein surface coverage) was calculated from the difference between the amount of protein used to prepare an emulsion and that in the aqueous phase after centrifugation from the specific surface area determined by the particle-size analyzer.

Electron spin resonance (ESR) analysis. Droplet surfaces of creams were spin-labeled by adding either 5-SA or 16-SA to the oil phase (0.25 mg per 1 mL of cream) before emulsification. The prepared emulsions with the spin probe were employed for the ESR determination (JES-RE2X; Nihondenshi, Tokyo, Japan). ESR spectra were recorded as follows: field: 3280 ± 50 Gauss; microwave frequency: 9.19 GHz; power: 10 mW. The correlation-relaxation time of the spin probe was derived from the Kivelson's formula and Freed's formula (using Brownian diffusion) by using the three main lines and hyperfine coupling constant $T_{\|}$, respectively (10–12).

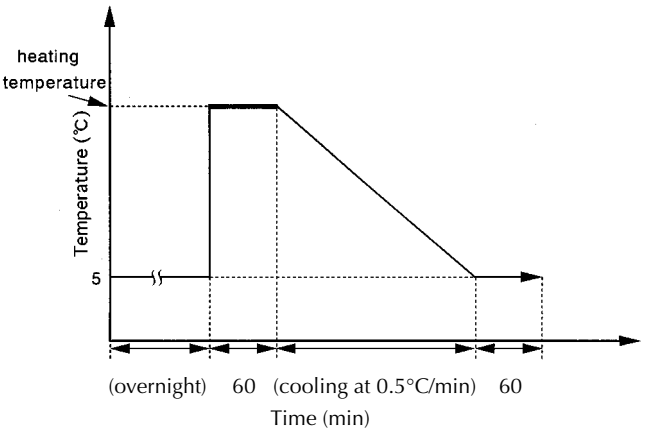

FIG. 1. Temperature-time profile for the cooling processes of creams after thermal treatment at various temperatures (heavy solid line).

RESULTS AND DISCUSSION

Particle size distribution of the prepared creams. Table 1 shows the data on oil-droplet particle-size distributions of the prepared creams. Particle sizes of prepared creams were relatively homogeneous, and their average diameter and distribution range were about 1 μm and 0.1–10 μm, respectively. Thermal treatment did not influence the oil-droplet size distributions of the creams (data not shown).

Change of SFC of creams. As described in the introduction, it is known that thermal treatment at temperatures that generate SFC in the range from a small percentage to 10% in the oil droplets causes coagulation or a viscosity increase of creams after a recooling process. Therefore, to find out the temperatures that induce a small percentage of SFC in the oil

TABLE 1
Average Droplet Diameter (d_{32}) and Specific Surface Area of Creams at 5¡C Before Thermal Treatment

	d_{32} (μm)	Specific surface area (cm^2/cm^3)
Cream A	1.88 ± 0.13	36,900 ± 4,200
Cream B	1.63 ± 0.12	44,800 ± 3,600

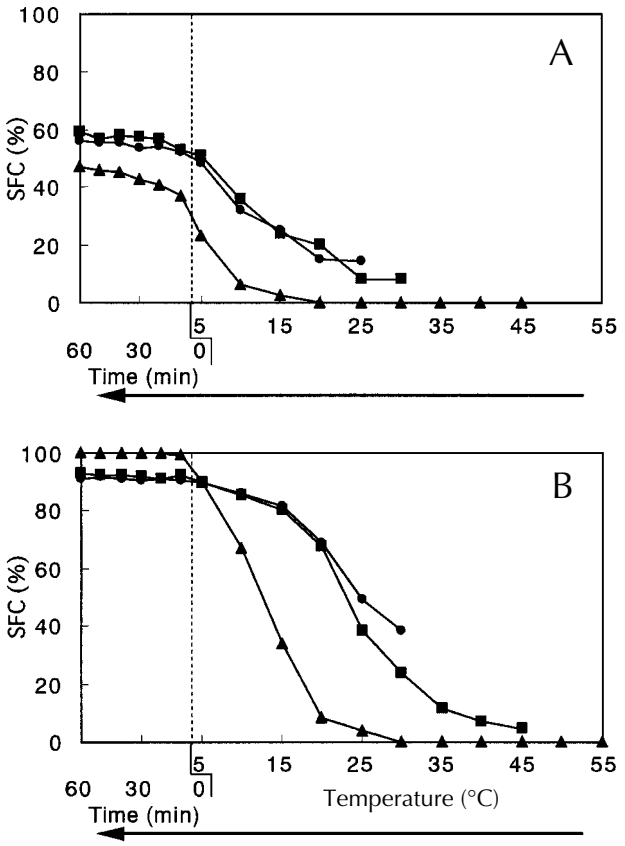

FIG. 2. Solid fat content (SFC) values of oil droplets in creams as a function of temperature. The arrow indicates the direction of temperature change, i.e., creams were cooled from the set temperature of thermal treatment to 5°C, and were kept at 5°C for 60 min as shown in Figure 1. (A) Cream A: ●, ■, and ▲ indicate the cooling processes after thermal treatment at 25, 30, and 45°C, respectively. (B) Cream B: ●, ■, and ▲ indicate the cooling processes after thermal treatment at 30, 45, and 55°C, respec-

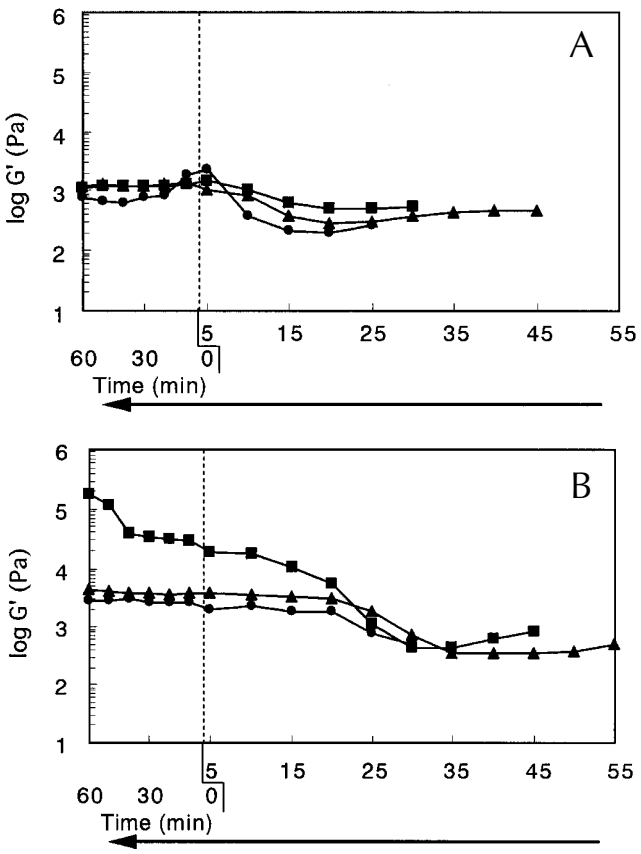

FIG. 3. Storage modulus (G') of creams as a function of temperature. (A) Cream A; (B) Cream B. The meanings of arrow and symbols are the same as those of Figure 2.

droplets (the so-called "critical temperature" in the following sentences) in the prepared creams, SFC measurements were carried out for the creams that had been incubated at various temperatures in the range of 25–55°C, followed by cooling to 5°C. Figure 2 shows the SFC values of oil droplets in creams as a function of temperature. The "critical temperatures" of cream A and cream B were 30 and 45°C, respectively. Mutoh *et al.* (8) reported similar results for creams including fats A and B, although the composition of their creams was more complex and resembled that of commercial creams, that is, the "critical temperature" mentioned above for cream A was not 30 but 35°C. Thermal treatment of the creams at the critical temperatures induced a rapid increase of SFC in both creams during the cooling process. This may be attributed to heterogeneous nucleation of fats in the oil droplets. On the other hand, thermal treatment of the creams at temperatures above the critical values, i.e., 45 and 55°C for creams A and B, respectively, retarded the onset of SFC increase during the cooling process. In this situation, homogeneous nucleation of fats in the oil droplets may be dominant in the cooling process, which led to supercooling (1). Such supercooling phenomena correspond with the data of Dickinson *et al.* (13,14), who used a single kind of triacylglycerol. The SFC values were 20 and 40% for creams A and B, respectively, when the creams were treated at temperatures below critical, i.e., 25 and 30°C. During the cooling process, however, SFC curves approached the curves of samples that were treated at critical temperatures. So, this may also be attributed to heterogeneous nucleation of fats in the oil droplets.

Viscoelastic measurements of thermally treated creams. Figure 3 shows the storage modulus (G') of creams during the cooling process after thermal treatment. Because of water evaporation and subsequent solidification of creams at the edge of the plate during heating for an hour, the baseline of G' increased to the level of 10^2 Pa. For cream B, the thermal treatment at 45°C induced a dramatic increase of G' during the cooling process, suggesting aggregation of oil droplets. Such flocculation was confirmed by the change of droplet size distribution (data not shown). The sudden increase started especially in the range from 25 to 20°C. Thermal treatment of cream B at 30 and 55°C did not cause a dramatic increase of G' in the following cooling process. The dramatic increase of G' was only observed for thermal treatment at the "critical temperature," i.e., 45°C. This means that heating of the cream at the "critical temperature" was essential for the increase of viscosity during the cooling process.

For cream A, however, all heating conditions, even heat-

ing at the "critical temperature" (30°C), could not cause a rapid increase of G' during the cooling process. Contradictory to our results, Mutoh *et al.* (8) found that the viscosity of the cream that contained fat A increased during the cooling process after thermal treatment at the "critical temperature." On the other hand, heating at temperatures above and below the "critical" value could not cause an increase in viscosity of the cream. The simple composition of cream A, which contained only β-casein as an emulsifier, may be responsible for no increase in viscosity of cream A (Fig. 3A).

When soybean or olive oils, which stay liquid in the temperature range of Figure 1, were used for preparation of creams, such creams were stable after thermal treatment followed by the cooling process (data not shown).

Changes in amount of adsorbed protein at the oil droplet surface. Figure 4 shows the protein surface coverage as a function of temperature. For cream A, the protein surface coverage was almost constant during the cooling process for all thermal treatments. For cream B, however, a dramatic increase of protein surface coverage was observed during the cooling process, especially below 25°C, when the cream was thermally treated at 45°C. This change of protein surface coverage was not caused by the change of specific surface area of cream B, because oil-droplet size distributions of cream B,

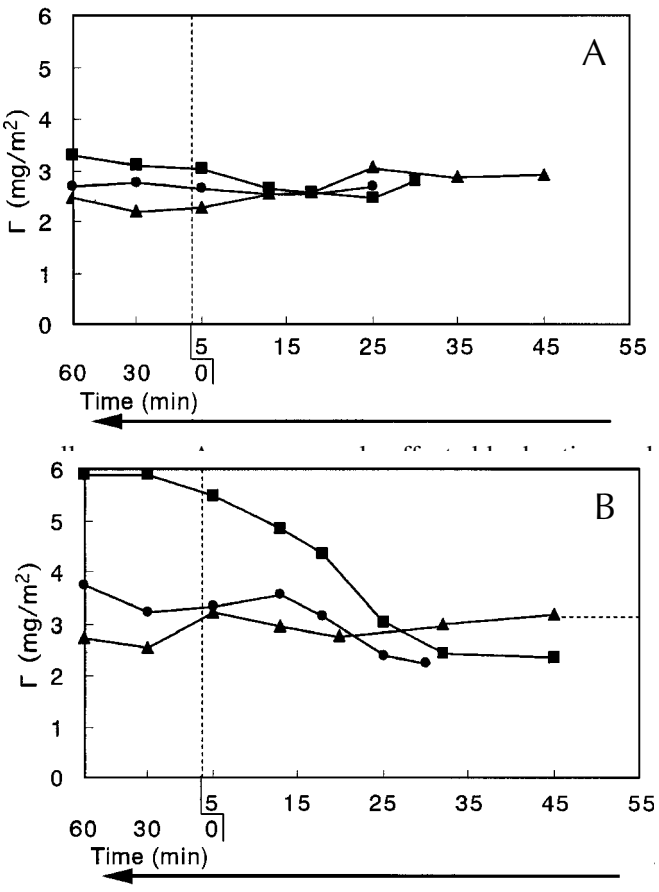

FIG. 4. Protein surface concentration (Γ) at oil droplet of creams as a function of temperature. (A) Cream A; (B) Cream B. The meanings of arrow and symbols are the same as those of Figure 2.

cream B without dilution at 45°C caused the aggregation of oil droplets and a viscosity increase during the cooling process (Fig. 3B). These results suggest a close relationship between the increase of protein surface coverage and the flocculation of oil droplets (and subsequent increase of viscosity of cream B), although the diluted cream did not flocculate under the same conditions.

Similar results were obtained for α-lactalbumin-stabilized creams with respect to the change of protein surface coverage as a function of temperature (data not shown). In particular, the dramatic increase of protein surface coverage was only observed for cream B that was thermally treated at 45°C and cooled. The final value at 5°C was 6 mg/m^2, the same as for β-casein (data not shown). Therefore, we believe that the adsorption behavior of protein in thermally treated and cooled creams is independent of the kind of protein used.

When liquid oils (soybean or olive) were used for the preparation of creams, the protein surface coverage did not change with thermal treatment and subsequent cooling (data not shown).

Fluidity of lipid molecules at the oil-droplet surface. To investigate the change in fluidity of lipid molecules at the oil droplet surface as a function of temperature, ESR analyses of the creams containing spin probes, such as 5-SA and 16-SA, were carried out, and rotational correlation times (τc) of spin probes (5-SA; 16-SA) on the oil-droplet surfaces were calculated by means of Kivelson's formula (10,11).

In the pattern of 16-SA, shown in Figure 5, τc values increased gradually and continuously for all samples during the cooling process. These results suggest that the mobility of the lipid molecule part situated at a position a little farther away from the droplet surface (depth of hydrocarbon chain consisting of 16 carbon atoms) did not change drastically with the decrease of temperature.

For 5-SA, where the radical is situated at a position close to the oil-droplet surface (depth of hydrocarbon chain consists of five carbon atoms), the increasing pattern of the τc value differed depending on the heating conditions and kinds of creams. For cream A, the τc value did not change greatly with the decrease of temperature, although a slight increase started at 10°C during the cooling process. For cream B, however, a dramatic increase of τc value during the cooling process was observed when the cream was treated at 45 or 30°C. Because increases of viscosity (Fig. 3) and the protein surface coverage (Fig. 4) during the cooling process were only observed for cream B, the dramatic decrease of τc for cream B suggests that the lowering of fluidity of the lipid molecules at the oil-droplet surface is closely related to the aggregation of oil droplets, accompanied by further adsorption of proteins onto the oil-droplet surfaces.

For cream B, the sudden increase of τc value started at 20°C when the thermal treatment was carried out at 45°C. This temperature (20°C) corresponds to the onset temperature of oil-droplet aggregation and further adsorption of proteins for cream B treated at 45°C. The dramatic reduction of fluidity of lipid molecules at 20°C may be essential for the

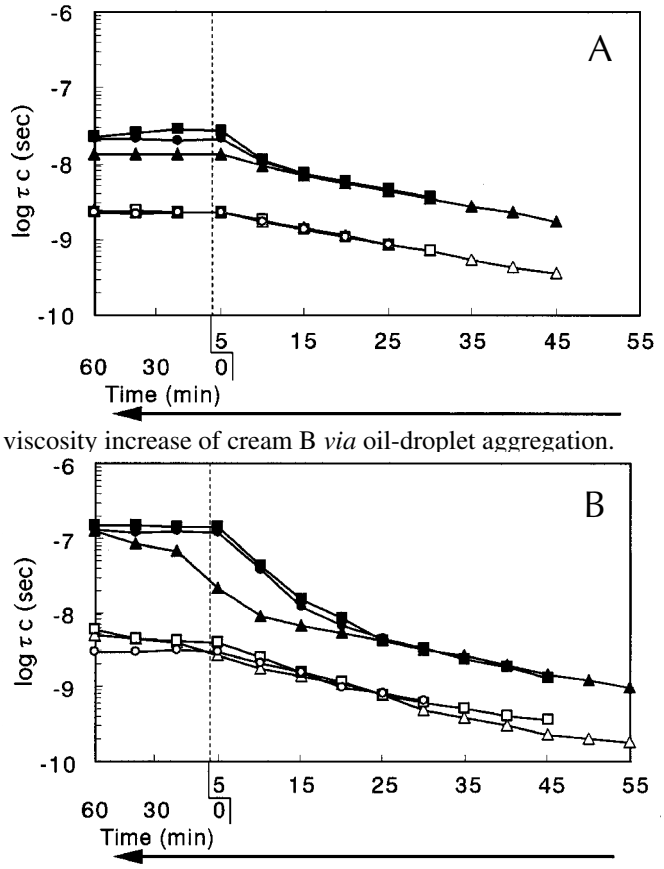

FIG. 5. The correlation-relaxation time (τc) at oil-droplet surfaces of creams as measured with 5-SA (solid symbol) and 16-SA (open symbol). (A) Cream A; (B) Cream B. The meanings of arrow and symbols are the same as those of Figure 2. Abbreviations: 5-SA and 16-SA, 5- and 16-doxyl (4,4-dimethyloxazolidine-*N*-oxyl)-stearic acid.

tial but not sufficient factor for the destabilization of cream.

Though the τc value derived from the Kivelson's formula is correct when it is less than 10^{-9} s, the value is not reliable when this formula is applied to the calculation of τc values larger than 10^{-9} s. Therefore, for $\tau c > 10^{-9}$ s, Freed's formula was adopted instead of Kivelson's (12). We found the same tendency for the results of the temperature dependence of τc, i.e., the dramatic increases of τc were only observed at 20°C in the cooling process when cream B was thermally treated at 45 or 30°C, although the values from Freed's formula did not agree with those from Kivelson's formula (data not shown).

ESR measurements were also performed with creams prepared from liquid oils (soybean and olive). The τc values of 5-SA and 16-SA in both creams gradually increased without dramatic jumps during the cooling process when the creams were treated at various temperatures. These results suggest that sudden changes in fluidity of liquid molecules at the oil-droplet surface did not occur with the decrease of temperature for these creams.

Based on our results, we propose the following mechanism for the destabilization of cream prepared from oils that consist of various kinds of triacylglycerols with different melting points. When cream B is thermally treated at 45°C for 1 h, about 4.4% of fat crystals are generated in the oil droplets and approach the oil-droplet surface, thereby causing a conformational change of the protein molecule adsorbed at the oil-droplet surface. During the following cooling process, fluidity of the triacylglycerol molecules at the oil-droplet surface decreases with an increase of the SFC in the oil droplets. The rapid decrease of fluidity of triacylglycerol molecules at the oil-droplet surface observed at 20°C is of great importance. At this temperature, probably, the conformational change of protein is more enhanced, and the zeta potential of the oil-droplet surface also changes slightly (Sugimoto, T., Y. Matsumura, and T. Mori, unpublished data). As a result, proteins dissolved in the dispersing medium are attracted to the adsorbed proteins at the oil-droplet surface (or adsorbed onto the space among protein molecules at the oil-droplet surfaces). The aggregation of oil droplets takes place *via* such further adsorption of proteins from the aqueous medium. In this manner, the viscosity increase and the solidification of the cream may finally occur.

After thermal treatment of cream B at 55°C, no fat crystals are produced in the oil droplets. This situation does not affect the conformation of adsorbed proteins unlike after thermal treatment at 45°C. Furthermore, when cream B is thermally treated at 55°C, the rapid decrease of fluidity of triacylglycerol molecules at the oil-droplet surface started at 5°C, which is lower than the 20°C measured after thermal treatment at 45°C. Therefore, the viscosity increase cannot be observed when cream B is thermally treated at 55°C.

When cream B is heated at 30°C, 40% of fat crystals are generated, and more crystals approach the oil-droplet surface than after thermal treatment at 45°C. The conformational change of adsorbed protein in this situation at 30°C should be different from that at 45°C. This may be the reason for no further adsorption of protein from the aqueous medium and no oil-droplet aggregation in the cooling process. Therefore, at this stage, we can speculate that the slight conformation change of proteins adsorbed at the oil-droplet surface at 45°C is an important factor for the oil-droplet aggregation and the viscosity increase of cream B. Our preliminary experiments (Sugimoto, T., Y. Matsumura, and T. Mori, unpublished data) with enzymatic proteolysis suggest that the conformational change of adsorbed β-casein at the oil-droplet surface in cream B is caused by the thermal treatment at 45°C (data not shown). Rosenthal *et al.* (3,4) also found that the adsorption and interaction of sodium caseinate were enhanced by fat crystals present in the oil phase by using a surface rheological technique. Further investigation with NMR, ESR, and proteolytic methods is needed to understand the conformation and the physical states of proteins adsorbed at the droplet surface (15–19).

For cream A, the lack of a dramatic decrease of fluidity of triacylglycerol molecules at the oil-droplet surface during the cooling process may be the reason for no solidification of cream A accompanied by oil-droplet aggregation.

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