Destabilization of protein-based emulsions caused by bacteriostatic emulsifiers

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CONTENTS

ABBREVIATIONS

MW	Molecular weight
SDS	Sodium dodecyl sulfate
PAGE	Polyaclylamide gel electrophoresis
ANOVA	Analysis of variance
C10	$C_{10}H_{22}$ (Decane)
C12	$C_{12}H_{26}$ (Dodecane)
C14	$C_{14}H_{30}$ (Tetradecane)
C16	$C_{16}H_{34}$ (Hexadecane)
C18	$C_{18}H_{38}$ (Octadecane)
DF	Diglycerol esters of different mono-saturated or unsaturated fatty acids
DL	Diglycerol esters of mono-lauric acid
DM	Diglycerol esters of mono-myristic acid
DP	Diglycerol esters of mono-palmitic acid
DS	Diglycerol esters of mono-stearic acid
DO	Diglycerol esters of mono-oleic acid
MO	Monoglycerol esters of mono-oleic acid
TO	Triglycerol esters of mono-oleic acid
Na-CN	Sodium caseinate
HLB	Hydrophile-lipophile balance

General Introduction

A great variety of food products, natural or manufactured, exist as emulsions; for example, milk, cream, fruit juice, dressing and margarine (Dalgleish, 2004; McClements, 2005). Emulsions consist of two immiscible liquids, which are usually oil and water in the case of food products. Although the two phases essentially separate to form a plane interface system rapidly, surface-active molecules defined as emulsifiers, e.g., proteins and small-molecule amphiphilic agents adsorb to the droplet surfaces to stabilize the emulsion system mainly during emulsification processes (Kralova & Sjöblom, 2009).

Milk, originally an oil-in-water emulsion and its ingredients are often added to fruit juice, coffee or tea drinks to produce milk beverages, one of the most consumed products in the world (Molina, Amigo & Quirós, 2009). Such commercial products are often manufactured in packed, canned or bottled state with heat treatments to ensure the long-term shelf-life on a display cabinet or in a refrigerator in retailing stores. These liquid-type food emulsions undergo time-dependent undesirable changes in their properties such as appearance, texture and flavor due to physical, biological and chemical processes (McClements, 2005). Controlling and predicting these types of changes during the long-term storage are important concerns to researchers and manufactures in the food science field. In particular, physical instability of emulsions usually attracts their marked attention because emulsions are thermodynamically unstable and thereby destabilization process is essentially inevitable (Dickinson, 1992).

Emulsions separate into oil and aqueous phases normally via destabilizing processes such as creaming, flocculation, aggregation, and coalescence. For the milk-based emulsions including canned or bottled coffee/tea with milk, creaming and aggregation of oil droplets in the emulsion are often observed and tend to become a critical problem in the long-term shelf-life. Creaming and

aggregation can be perceived as white ring or float at the top of vessels and grainy fat globules on the surface of the beverage, respectively (Dickinson, 1994), which may not be accepted by most of the consumers due to poor appearance or uncomfortable mouth feel .

Milk itself, even the powdered one dispersed in water, is empirically known to be kinetically stable to a satisfactory extent under low-acidity conditions in milk beverages, since milk proteins such as caseins and whey proteins suspended in aqueous phase or adsorbed to oil droplets are effectively separated or well-dispersed through charge repulsion (Dickinson, 1998; Muthukumarappan & Karunanithy, 2009). Milk beverages, however, are quite often destabilized by addition of emulsifiers with bacteriostatic effects which are necessarily added to inhibit the growth of heat-resistant sporeformers in an anaerobic container (Kabara, Swieczkowski, Conley & Truant, 1972; Kato & Shibasaki, 1975). The mechanism whereby such emulsifiers destabilize milk beverages is still unclear. To compensate the destabilizing effects of the emulsifiers, manufactures practically apply stability-enhancing emulsifiers into the beverages in combination with the bacteriostatic emulsifiers. Since the stability of the milk beverages varies with combinations of two kinds of emulsifiers, that is, bacteriostatic and stability-enhancing emulsifiers, manufacturers have to determine the optimal formulation of the emulsifiers by performing trial-and-error type tests about the long-term stability of the milk beverages. Since this situation is a great disadvantage for the development of new products, the rapid and accurate evaluation method concerning the emulsion stability is highly required (Das & Kinsella, 1990).

In this context, this work deals with emulsion stability including creaming and aggregation of milk fat globules in canned milk coffee including the bacteriostatic and stability-enhancing emulsifiers in order to analyze factors affecting the creaming rate and extent of aggregation (Chapter 1). These instability phenomena observed in the milk-based emulsions mainly depend on the size of oil droplets and different interfacial properties between oil and water, i.e., thickness,

rheology and electric charge on the oil droplet surfaces (Wilde, 2000; Dickinson, 2001). The author investigates major factors that determine the characteristics of the interfacial region for the first step.

Diglycerol esters of fatty acids, a representative of the bacteriostatic emulsifiers used for the canned beverages promotes not only creaming and aggregation but also coalescence of oil droplets stabilized by milk proteins under certain conditions (Holstborg, Pedersen, Krog & Olesen, 1999). As a systematic and fundamental approach to clarify relationships between the structure and the destabilizing effects of the emulsifiers concerning oil phase types, a wide variety of polyglycerol esters of fatty acids with different polymerization of glycerols and various chain lengths of fatty acid residues are provided. The author focuses on effects of the molecular structural similarity between oil phase and fatty acid residues of the emulsifiers (Chapter 2), and the size of polar head of the emulsifiers (Chapter 3) on the destabilization of the protein-based emulsions.

Lastly, the author develops a novel method for rapid determination of aggregation forces between colloidal particles such as emulsion oil droplets and suspension particles to evaluate the long-term storage of the milk beverages rapidly and simply (Chapter 4). The central idea of the newly developed method is that particles with stronger aggregation forces tend to form aggregates and should not be redispersed easily.

REFERENCES

Dalgleish, D. G. (2004). *Food Emulsions*, fourth edition, revised and expanded. Friberg, S. E. Larsson, K. & Sjöblom, J., Eds. Marcel Dekker, New York, USA. pp.1-44.

Das, K. P. & Kinsella, J. E. (1990). *Advances in Food and Nutrition Research*, vol 34. Academic Press Inc., London, UK. pp. 81-202.

Dickinson, E. (1992). *An Introduction to Food Colloids*. Oxford University Press, Oxford, UK. pp. 140-173.

Dickinson, E. (1994). Colloidal aspects of beverages. *Food Chemistry* 51, 343–347.

Dickinson, E. (1998). Stability and rheological implications of electrostatic milk protein-polysaccharide interactions. *Trends in Food Science & Technology* 9, 347-354.

Dickinson, E. (2001). Milk protein interfacial layers and the relationship to emulsion stability and rheology. *Colloids and Surfaces B: Biointerfaces* 20, 197–210.

Holstborg, J., Pedersen, B.V., Krog, N. & Olesen, S.K. (1999). Physical properties of diglycerol esters in relation to rheology and stability of protein-stabilised emulsions. *Colloids and Surfaces B: Biointerfaces* 12, 383-390.

Kralova, I. & Sjöblom, J. (2009). Surfactants used in food industry: A review. *Journal of Dispersion Science and Technology* 30, 1363-1383.

Kabara, J.J., Swieczkowski, D.M., Conley, A.J. & Truant, J.P. (1972). Fatty acids and derivatives as antimicrobial agents. *Antimicrobial Agents Chemotherapy* 2, 23-28.

Kato, N. & Shibasaki, I. (1975). Comparison of antimicrobial activities of fatty acids and their esters. *Journal of Fermentation Technology* 53(11), 793-801.

McClements, D.J. (2005). *Food Emulsions: Principles, Practice, and Techniques,* second ed. CRC

Press, Boca Raton, Florida. pp. 1-26.

Molina, E., Amigo, L. & Quirós, A. (2009). *Milk Processing and Quality Management*. Blackwell

Publishing Ltd., Oxford, UK. pp. 294-316.

Muthukumarappan, K. & Karunanithy, C. (2009). *Handbook of Dairy Foods Analysis*, CRC Press,

Boca Raton, Florida. pp. 603-614.

Wilde, P. J. (2000). Interfaces: their role in foam and emulsion behavior. *Current Opinion in Colloid*

& Interface Science 5, 176-181.

Chapter 1:

Effects of bacteriostatic emulsifiers on stability of milk-based emulsions

1. INTRODUCTION

Milk is one of the most important materials in the food industries because it is processed to create a wide variety of food products, for example, ice cream, butter, milk powder, cheese and yogurt (McClements, 2005). Milk is also used as an ingredient of various beverages. For example, coffee, tea and fruit juice are often mixed with milk to impart a desirable flavor to them. In its natural state, milk is an oil-in-water emulsion in which fat globules are dispersed in an aqueous phase including a complex mixture of proteins, sugars and salts (McClements, 2005). Milk powder, which is prepared after the drying process of milk, returns to the emulsion state after dispersing it into water, although the original structure of the fat globules are possibly modified by the drying process (Walstra, Wouters & Geurts, 2006). Powdered milk, as well as fresh milk, is used very often for the production of canned coffee and tea.

Though milk originally includes surface-active molecules such as proteins (caseins and whey proteins) and phospholipids (Walstra *et al.*, 2006), canned coffee or tea that includes fresh milk or powdered milk is normally combined with small-molecule food emulsifiers. The use of the emulsifiers is mainly aimed at providing bacteriostatic effects on heat-resistant sporeformers, which are naturally contained in the milk. Kabara, Swieczkowski, Conley & Truant (1972) and Shibasaki (1979) reported the bacteriostatic effects of fatty acids esters. In spite of the name "emulsifiers", these emulsifiers with bacteriostatic ability often promote destabilization of the emulsion. That is, an unsightly ring and a white accumulation of fat globules floating at the top of canned coffee or tea, which is often observed during its long-term shelf-life. The food industry copes with this problem by adding another type of emulsifier with an ability to enhance the emulsion stability in order to

compensate for the undesirable effects of the bacteriostatic emulsifiers. When both types of emulsifiers, that is, the bacteriostatic emulsifiers and the stability-enhancing emulsifiers are added to canned coffee or tea with milk, the product shows a different stability with respect to creaming or aggregation, according to the different combinations of emulsifiers. Since the mechanisms of the bacteriostatic emulsifiers to cause the emulsion destabilization are unclear and the prediction for the stability of canned coffee or tea that includes both types of emulsifiers is very difficult, manufacturers have to examine the long-term stability of the emulsion (for example from 3 to 6 months storage), when developing new commercial products. To predict the emulsion stability accurately and design the optimal formulation for canned coffee or tea with milk, manufactures should investigate the effects of both types of emulsifiers on the physicochemical properties of the emulsions stabilized by milk proteins.

Emulsion destabilization occurs through the following steps; creaming, flocculation and coalescence. The creaming rate is very sensitive to droplet-size distribution. For an oil-in-water emulsion prepared using milk proteins, it has been shown that the initial droplet-size distribution is a crucial factor for the emulsion shelf-life (Dickinson, 2001).

Adsorbed protein molecules at oil droplet surfaces prevent the flocculation of oil droplets via steric hindrance and electrostatic repulsion (Bos & van Vliet, 2001; Tcholakova, Denkov, Ivanov & Campbell, 2002). Adsorbed proteins also contribute the formation of a cohesive layer, which is resistant to coalescence of oil droplets. However, small-molecule emulsifiers are found to be capable of displacing adsorbed proteins from the oil-water interface thereby affecting the emulsion stability (De Feijt, Benjamins & Tamboer, 1987; Dickinson, Euston & Woskett, 1990; Courthaudon).

The aim of the present study is to understand the reason why the stability of milk-based emulsion changes according to the combination of bacteriostatic and stability-enhancing emulsifiers

from the viewpoint of the interaction between milk proteins and emulsifiers at the oil droplet surface. To accomplish this purpose, we prepared four milk-based emulsions with varying stability, from powdered milk and emulsions, and compared the amount and composition of adsorbed protein at the oil droplet surface. The particle size distribution and ζ-potential (the index of electric repulsive force) of oil droplets were also measured in order to assess the contribution of these factors to the emulsion stability. The amount of phospholipids was measured, since phospholipids are included in powdered milk and thought to affect its emulsion stability.

2. MATERIALS AND METHODS

2.1 Materials

All general chemicals used were of analytical grade purchased from Nacalai Tesuque, Inc. (Kyoto, Japan) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan).　Deionized water was used for the preparation of all solutions. Powdered milk was obtained from Snow Brand Milk Products Co., Ltd (Tokyo, Japan). P-1670 (sucrose palmitate, HLB 16), S-570 (sucrose stearate, HLB 5) and S-770 (sucrose stearate, HLB 7) were manufactured by Mitsubishi-Kagaku Foods Corporation. Of these emulsifiers, DP-95 (diglycerol esters of palmitic acid, HLB 8) and BS-20 (succinate mono-glyceride, HLB 5.5) were given to us by Riken Vitamin Co., Ltd. DP-95 and P-1670 are bacteriostatic emulsifiers, while BS-20, S-770 and S-570 are stability-enhancing emulsifiers. Powdered milk and all the emulsifiers were stored at 4 °C prior to their use.

2.2 Emulsion preparation

Compositions of the emulsions are shown in Table 1-1. The powdered milk-based emulsion was prepared according to the following procedure. An emulsifier solution was prepared by homogenizing the emulsifiers in the appropriate amount of boiling water using a high-speed blender (Physcotron, NS-51, Microtec Co., Ltd.) at 19,300 rpm for 2 min. Powdered milk was dispersed into the appropriate amount of boiling water using the blender at 19,300 rpm for 2 min, and the

Table 1-1. Compositions of milk-based emulsions

	Control	Composition 1	Composition 2	Composition 3
Powdered Milk	5.0 g	5.0 g	5.0 g	5.0 g
DP-95		893 mg	1250 mg	
P-1670				1259 mg
BS-20		536 mg	446 mg	
S-770				714 mg
S-550			446 mg	

(in 100ml water)

resultant solution was then blended with the emulsifier solution at 19,300 rpm for 3 min using the blender. The reason why the author used boiling water is that the usage of boiling water reflected practical manufacture of the canned coffee. The powdered milk-based emulsion was cooled down in ice water for 3 min, and then water was added to adjust the final concentration of powdered milk to 5.0% (w/v). The resulting emulsion was autoclaved at 121 °C for 30 min (EYELA, MAC-601, Tokyo Rikakikai Co., Ltd) for sterilization, and then stored overnight at 4 °C. We produce canned coffee under this condition. The four emulsions of the same composition including coffee extract were prepared at a pilot scale in the laboratory of Pokka Corporation. The emulsion containing coffee (coffee emulsions) were stored in can for 3 months at 4 °C in order to test long-term stability. On the other hand, the four emulsions without coffee extract were used the following experiments.

2.3 Particle size analysis

The particle size of the prepared emulsion was measured before and after the heat treatment by the autoclave. The measurement was also carried out after the overnight storage. The prepared emulsion was diluted to an appropriate concentration using water in order to avoid multiple scattering and then the particle size distribution was measured using a laser diffraction instrument (Particle Size Analyzer LA-500, Horiba Ltd., Japan). The particle size measurements were reported as the volume-average mean diameter, $d_{3,2}$ (=$\sum f_i d_i^3 / \sum f_i d_i^2$, where f_i is the frequency of particles with diameter d_i).

2.4 ζ-Potential measurements

ζ-Potential of oil droplets is determined using a particle electrophoresis instrument (ELS-Z1, Photal, Otsuka Electronics Co., Ltd., Japan). The powdered milk-based emulsions were diluted 2000-fold using deionized water prior to measurements, and were then taken into the chamber of the instrument.

2.5 Isolation of oil droplets from the powdered milk-based emulsion

Oil droplets in emulsions were isolated to measure the adsorbed or incorporated proteins and phospholipids according to the method of Dickinson & Tanai (1992) and Euston, Singh, Munro & Dalgleish (1995). The powdered milk-based emulsion was centrifuged at 140,000g for 40 min at 4 °C using an ultracentrifuge (CP-75β, Hitachi Koki Co., Ltd., Japan) in order to separate an opaque cream layer at the top, a transparent layer in the middle, and cloudy brown precipitation at the bottom. The cream layer at the top was transferred into another sample tube, and was combined with the appropriate amount of deionized water. The cream was dispersed well in the water through vigorous shaking using a vortex mixer. The resultant emulsion was then centrifuged at 87,000g for 30 min at 4 °C using the ultracentrifuge to separate it into an opaque cream layer at the top and transparent layer at the bottom. The washed cream layer was subjected to the same procedure to wash it again. The cream layer, separated again, was gathered and transferred into another sample tube, and then freeze-dried. The dried cream was weighed, half of which was used for the determination of proteins, and the other was used for the determination of phospholipids.

2.6 Measurements of phospholipids in the oil droplets

Lipid was extracted from the dried cream by the modified method of Bligh & Dyer (1959). To the dried cream (approximately 100-200 mg), 1.5 ml of water, 2.5 ml of methanol and 1.25 ml of chloroform were added and then stirred. The mixture was stored for 10 min at room temperature, followed by the addition of 2.5 ml of chloroform and 2.5 ml of 1 wt% NaCl aqueous solution. The

mixture was centrifuged at 950g for 5 min at 4 °C using a centrifuge (CR-5B, Hitachi Koki Co., Ltd., Japan). It was separated into a water-methanol layer at the top, a fluff layer in the middle and chloroform layer at the bottom. The chloroform layer was transferred to another test tube, and combined with a small amount of Na_2SO_4. After the storage overnight at 4 °C, the sample was centrifuged again in the same condition. The chloroform layer was transferred to another test tube, and then was evaporated under a gentle N_2 stream.

The extracted lipids were dissolved into 3 ml of chloroform, which was used as sample solution. The concentration of phosphorus in the solution was determined using the Bartlett's method (1959) and KH_2PO_4 as a standard. The amount of phospholipids in the oil droplets was calculated using the average molecular weight (MW = 764) of phospholipids (Walstra, Wouters & Geurts, 2006).

2.7 Measurements of proteins adsorbed at the oil-water interface

The dried cream (which was obtained according to the method in the Section 2.5) was dispersed in 200 mM SDS-phosphate buffer (pH 7.0, 4 wt% SDS) and stored overnight at room temperature. The sample spontaneously separated into a cream layer at the top and an aqueous serum at the bottom. The serum was used for protein measurements. The protein concentration was determined using Lowry's method (Lowry, Rosebrough, Farr & Randall, 1951) and bovine serum albumin (BSA) as a standard. The amount of proteins adsorbed at the surface of oil droplets was expressed as surface concentration (mg/m^2), which was calculated using the data of protein concentration in the solution and the specific surface area. The specific surface area was estimated from the measured particle size, $d_{3,2}$ (McClements, 2005b).

2.8 Composition of adsorbed proteins

Compositions of adsorbed proteins were determined by SDS-PAGE with 2-mercaptoethanol. The concentration of acrylamide was 20 wt%. The dried cream was dispersed in

the electrophoresis sample buffer (pH6.8, Tris-HCl, 4 wt% SDS) and stored overnight at room temperature. The sample spontaneously separated into a cream layer at the top and an aqueous serum at the bottom. The serum layer at the bottom was applied onto 20 wt% of acrylamide gel. Each protein was then identified by comparing the mobility of its band with that of the purchased pure proteins.

2.9 Statistical analysis

All experiments were conducted in more than triplicate with freshly prepared emulsion. The effects of processing and emulsion composition on mean particle diameter were assessed using a two-way repeated-measures ANOVA, with processing (Before heat treatment/ After heat treatment/ After 24h storage) as one factor and emulsion composition (Control/ Composition 1/ Composition 2/ Composition 3) as the other. The two-way ANOVA was performed for the particle size analysis (Figure 1-2) to determine whether there was a significant effect of processing, or a significant effect of emulsion composition. The effect of emulsion composition on ζ-potential of oil droplets, amount of phospholipids in oil droplets and amount of proteins at the oil droplet surface were assessed using a one-way analysis of variance (ANOVA). The one-way ANOVA was performed separately for each experiment (Figures 1-3 to 1-5) to determine whether there was a significant effect of emulsion composition. The level for statistical significance was set $p < 0.05$ for all statistical analyses. Statistical analysis was performed using Microsoft Excel 2003 for Windows.

3. RESULTS & DISCUSSION

3.1 Long-term stability of milk-based emulsions including coffee extract

In order to test the stability of four emulsions containing coffee extracts, the emulsions were stored for 3 months in cans at 4 °C. Figure 1-1 shows the appearance of the emulsions after 3 months of storage. The control emulsions without emulsifiers showed a little white ring and floating
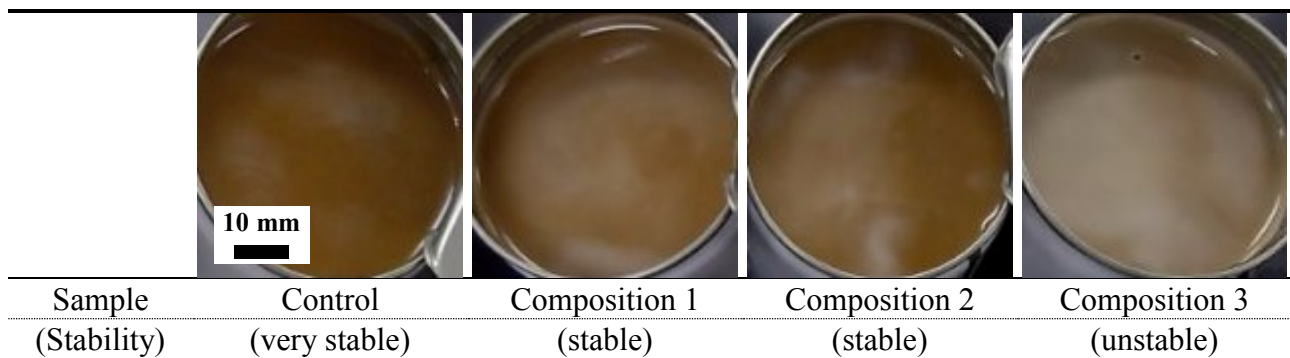
Sample (Stability)	Control (very stable)	Composition 1 (stable)	Composition 2 (stable)	Composition 3 (unstable)

Figure 1-1. Long-term stability of milk-based emulsions including coffee extracts.

After addition of coffee extracts, the emulsions were stored in cans. The stability was tested for three months at 4 °C by the appearance, that is, the occurrence of cream layer. The scale bar in the picture indicates 10 mm.

particles, indicating that this emulsion was very stable with respect to creaming or coalescence. For the emulsions of Compositions 1 and 2, a slight creaming was observed. However, the weak flocculation of this creaming layer was so weak as to be dispersible by shaking. Therefore, the author can judge that these emulsions are still stable. For the emulsion of composition 3, however, a white ring and floating particles were clearly observed, and these creaming products could not be dispersed by shaking, indicating high degree of coalescence/aggregation of oil droplets. From a commercial viewpoint, such irreversible coalescence/aggregation is not acceptable.

The following experiments (from Section 3.2 to 3.6) were carried out to understand the reason for the different stability of these four emulsions. The emulsions without coffee extract were used because coffee extract hampers or affects several experiments. We confirmed, however, that emulsion stability did not change significantly by the addition of coffee extract.

3.2 Influence of thermal processing and storage on particle size

The particle size of the milk-based emulsions was measured three times; after the preparation, after the heat treatment and after the overnight storage, in order to monitor the influence of heating process on the emulsion stability. The mean particle diameter is shown in
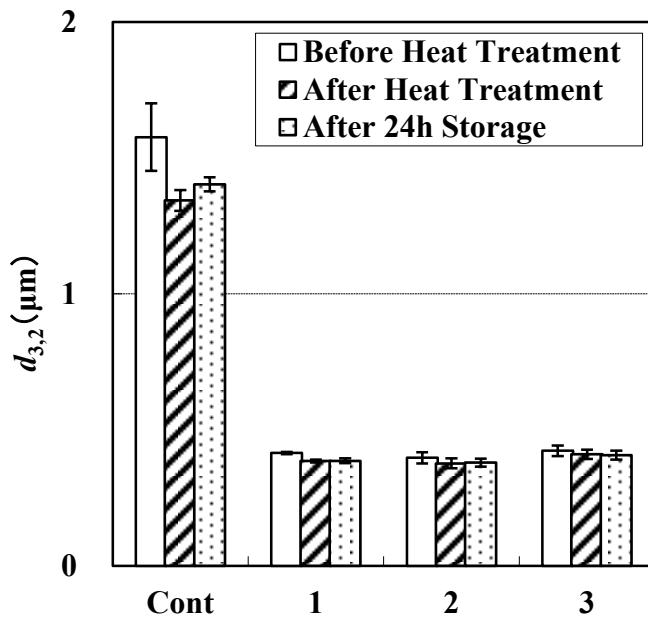
Figure 1-2. Changes in mean particle diameter ($d_{3,2)}$ of emulsions through heat treatment and storage.

Emulsions were subjected to heat treatment at 121 °C for 30 minutes followed by storing at 4 °C overnight. Particle size distribution of emulsions was measured just after the preparation, after the heat treatment and the storage overnight. Mean particle diameter was calculated from data of particle size distribution. The shape of particle-size distributions was mono-modal in all the samples.

Data was analyzed by using a two-way analysis of variance (ANOVA) with processing (Before heat treatment/ After heat treatment/ After 24h storage) and emulsion composition (Control/ Composition 1/ Composition 2/ Composition 3) as factors. The two-way ANOVA revealed significant main effects of processing ($p < 0.05$) and emulsion composition ($p < 0.05$). Cont: Control (very stable emulsion), 1: Composition 1 (stable one), 2: Composition 2 (stable one), 3: Composition 3 (unstable one).

Figure 1-2. The shape of particle size distributions was mono-modal in all the samples.

The particle size of the milk-based emulsion was significantly changed by the processing (thermal treatment and overnight storage at 4 °C) (two-way ANOVA, $p < 0.05$). But, the change was not so large and had little importance from the practical viewpoints. This shows that the emulsion state was not greatly affected by the heating process (sterilization and storage in cold temperature). Irrespective of heat treatment and storage at 4 °C, the mean particle diameter of the control ($d_{3,2}$= 1-2 μm) was far larger than that of Composition 1 ($d_{3,2}< 0.4$ μm), Composition 2 ($d_{3,2}< 0.4$ μm) and Composition 3 ($d_{3,2}< 0.4$ μm). Differential interference contrast microscopy revealed that there was

little flocculation of the oil droplets in the control (data not shown).

These results (Figure 1-2) show that small-molecule emulsifiers are necessary to create smaller oil droplets using the high-speed blender. Normally creaming velocity is closely related to the particle size of emulsions; that is, large droplets cream more rapidly according to Stokes' law. However, in the results of Figure 1-2, the mean particle diameter of the control was larger than that of the other emulsions, although the control emulsion showed the highest long-term stability as described above. Furthermore, the mean particle diameter of the unstable emulsion of composition 3 was similar to that of Compositions 1 and 2 emulsions. This suggests that the emulsion stability cannot be predicted by measuring the particle size of oil droplets and thus, other factors are responsible for the creaming stability in our case. Chanamai & McClements (2000) reported that the creaming velocity increased with flocculation because of the increase of the effective size of the oil droplets. In the following sections, several factors affecting flocculation of oil droplets were tested.

3.3 ζ -Potential measurements

The flocculation stability of the emulsion sometimes depends on the electrical properties of the droplets. The ζ -potential of the milk-based emulsion was measured to estimate the electrostatic repulsion between the droplets of the emulsion (Figure 1-3). The fact that the droplet charge was negative in the emulsion suggested that anionic molecules, e.g. caseins, β-lactoglobulin or negative-charged phospholipids, were adsorbed into the surface of the droplets. The ζ-potentials of all the emulsions were from -32mV to -35mV. Wade & Beattie (1997) compared the ζ-potentials of commercial milk and recombined milk (milk fat with commercial skim milk). Their ζ-potentials were -19 mV, and -36 mV respectively. The data obtained in this study (Figure 1-3) was similar to the ζ-potential value of the recombined milk emulsion, suggesting that the interfacial layer of the powdered milk-based emulsion has a similar structure to that of the recombined milk emulsion.
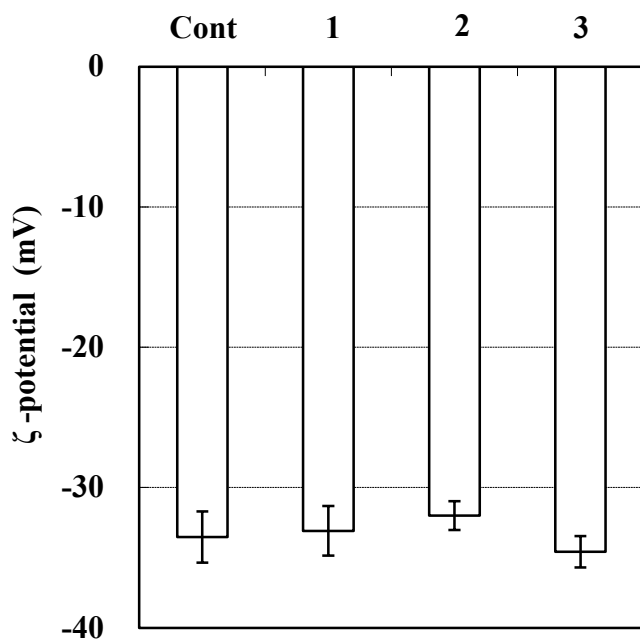
Figure 1-3. ζ- Potential of oil droplets in emulsions

Emulsions were diluted 2000 times and ζ-potential of oil droplets was measured after storage overnight at 4 °C. Data was analyzed by using a one-way analysis of variance (ANOVA) with emulsion compositions as factors. The one-way ANOVA revealed no effect of emulsion compositions. Cont: Control (very stable emulsion), 1: Composition 1 (stable one), 2: Composition 2 (stable one), 3: Composition 3 (unstable one).

The result shows that no significant difference was observed in ζ-potential among the control and the emulsions of Compositions 1 - 3, suggesting that the electrostatic repulsions did not play a dominant role in the stability of our milk-based emulsions. The ζ-potential values, -32 to -35 mV of the milk-based emulsions seem to be enough to keep oil droplets apart from each other (Friberg, Quencer & Hilton, 1996). Nevertheless, the reason why the creaming or flocculation occurs especially for the emulsion with compositions may be due to the composition and structure of interfacial layer of oil droplets. In the remaining sections, therefore, the experiments were performed on the behavior of surface active compounds, i.e., phospholipids and proteins.

3.4 Phospholipids in the oil droplets

Phospholipids, which were associated with the oil droplets, were measured to examine the relationship to the emulsion stability (Figure 1-4). Phospholipids are not only soluble in oil, but also dispersible in the water phase, and can be adsorbed to the oil-water interface because of their amphiphilic structure. Actually, the major phospholipids, lecithin (phophatidylcholine) was found to adsorb into the emulsion droplet surface and to displace the proteins. The values in Figure 1-4
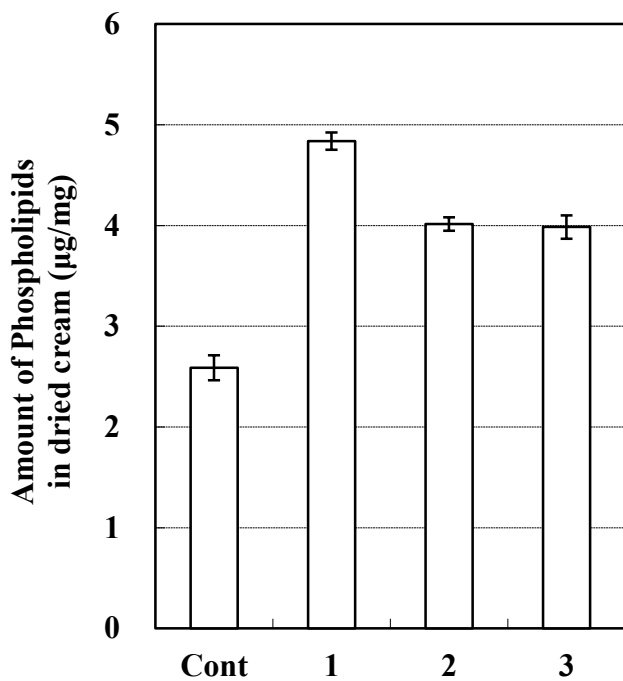
Figure 1-4. Amount of the phospholipids in oil droplets in emulsions

Cream layers of emulsions were separated by centrifugation, and total lipids were extracted from the collected cream. Content of phosphorus was determined by Bartlett's method. Amount of phospholipids was calculated from phosphorus content assuming that the averaged molecular weight of phospholipids is 764 and expressed as µg/mg dried cream. Data was analyzed by using a one-way analysis of variance (ANOVA) with emulsion compositions as factors. The one-way ANOVA revealed significant main effects of emulsion compositions ($p < 0.05$). Cont: Control (very stable emulsion), 1: Composition 1 (stable one), 2: Composition 2 (stable one), 3: Composition 3 (unstable one).

indicate the total phospholipids that were solubilized in the oil and adsorbed at oil droplet surface. The amount of phospholipids associated with oil droplets of the control, Compositions 1 - 3 emulsions were 2.6, 4.8, 4.0 and 4.0 µg/mg, respectively. The amount of phospholipid for the control emulsion (very stable) was comparatively lower than those of the other emulsions including emulsifiers. It is possible that the addition of the emulsifiers modify the adsorption behavior of phospholipids, thereby affecting the stability of emulsified oil droplets. It has been shown that phospholipids have the ability to change the structure of the adsorbed casein layer at the oil droplet surface (Fang and Dalgleish, 1993) and thus affect the emulsion stability (Matsumura, 2005).

Nevertheless, the author cannot perfectly explain the emulsion stability based on the amount of phospholipids associated with the oil droplets, because the amount for Composition 3 emulsion, with the lowest stability, was similar to that of Composition 2 emulsion. Probably, the increased amount of phospholipids may play an important role, but may not create a sufficient

condition to cause the destabilization of oil droplets in the emulsions.

3.5 Proteins on the droplet surface of oil droplets

Amounts of adsorbed proteins at oil droplet surfaces were measured to examine the competitive displacement between proteins and small-molecule emulsifiers (Figure 1-5). The values were expressed as protein surface concentration (mg/m^2). The amount of adsorbed proteins varied according to the composition of the emulsions. The amount of adsorbed proteins in the control emulsion was 14.5 mg/m^2, but the amount in the emulsion of Compositions 1 and 2 was decreased to 1.7 and 1.6 mg/m^2 respectively. The amount of adsorbed proteins in the emulsion of Composition 3 was the minimum, 0.6 mg/m^2. There was close relation, or correspondence, between the adsorbed protein and the stability of the emulsion, that is, the higher the amount of adsorbed protein, the higher the stability. This suggests that the adsorbed protein at the oil droplet surface may contribute to the emulsion stability via a steric hindrance mechanism. It is thought that the adsorbed protein layer in the control emulsion is thick enough to keep the oil droplets apart from each other, thereby preventing the aggregation of oil droplets. The thick layer of adsorbed proteins can also form the viscoelastic film which is resistant to the coalescence of oil droplets, another step of the destabilization process. The decreased amount of adsorbed proteins in the emulsions of Compositions 1 and 2 may lower the stability, but still be high enough to maintain the dispersion of oil droplets during storage. However, the amount of adsorbed protein in the emulsion of Composition 3 is too low to prevent the aggregation and coalescence of oil droplets. The decrease in the amount of adsorbed proteins is due to the ability of low-molecular weight emulsifiers to displace proteins at the oil droplet surface by the lowering interfacial tension (Wilde, Mackie, Husband, Gunning & Morris, 2004). Water soluble (or easily dispersible) emulsifiers with high HLB are apt to displace proteins more efficiently than those with low HLB (Dickinson *et al*., 1992). Since P-1670 is a sucrose ester with high HLB, it is reasonable that the amount of adsorbed proteins
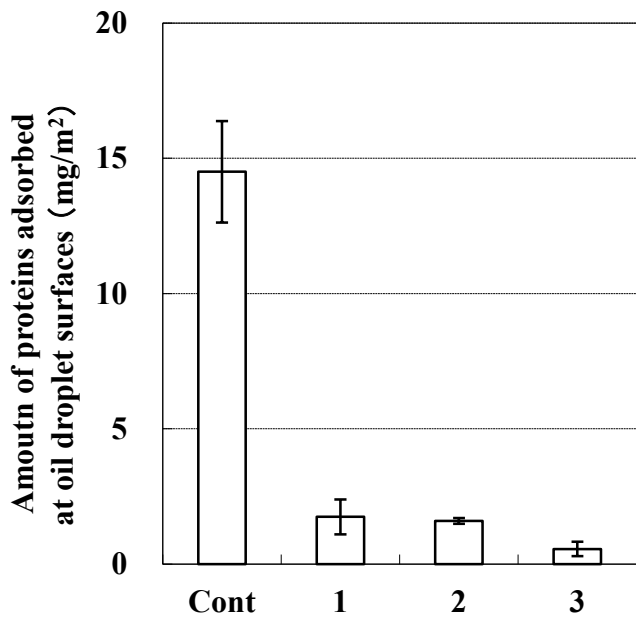
Figure 1-5. Amount of proteins at the oil droplet surface in emulsions

The separated cream layer from emulsions was dispersed in SDS-buffer to transfer adsorbed proteins from the oil droplet surface to the aqueous phase. Amount of desorbed proteins in SDS-buffer was determined by Lowry's method and expressed as mg/m2. Data was analyzed by using a one-way analysis of variance (ANOVA) with emulsion compositions as factors. The one-way ANOVA revealed significant main effects of emulsion compositions ($P < 0.05$). Cont: Control (very stable emulsion), 1: Composition 1 (stable one), 2: Composition 2 (stable one), 3: Composition 3 (unstable one).

was decreased to the minimum level in the emulsion of composition 3, which included this emulsifier. For emulsions of composition 1 and 2, DP-95 (diglycerol esters) was used to inhibit the activity of heat-resistant sporeformers, instead of P-1670. Since the surface activity of diglycerol esters type emulsifier is lower than that of emulsifiers with high HLB (Matsumiya, Takahashi, Nakanishi, Dotsu & Matsumura, 2007), DP-95 seems to be unable to displace proteins efficiently, and consequently may not affect the emulsions stability very much.

It is generally accepted in the food industry that the combination of proteins and oil soluble emulsifiers with low HLB is favorable for the production of fine emulsions with a high stability. Based on this knowledge, we can rationalize the Compositions 1 and 2 including BS-20 and S-550 with low HLB as a good formulation for producing stable emulsions. It is also likely that other mechanisms contribute to the stabilizing effects of BS-20 and S-550, such as modification of crystallization behavior in oil droplets by these emulsifiers (Mutoh, Kubouchi, Noda, Shiinoki & Matsumura, 2007).

3.6 Composition of adsorbed protein

The composition of adsorbed proteins at the oil droplet surface after 24-h storage was determined by SDS-PAGE with 2-mercaptoethanol (Figure 1-6). Since the presence of 2-Me scarcely affected the protein profile, the profile with 2-mercaptoethanol was only shown here (Figure 1-6). In the profile of control emulsions, many kinds of protein bands diffusing in the wide range of molecular weight were observed in addition to the main components such as α-, β-, κ-casein, β-lactoglobulin and α-lactalbumin. The total intensity of stained bands in the control profile was higher than that in the emulsion with Compositions 1 - 3. It was shown, in the previous section, that the amount of adsorbed proteins ranked in the following order; control > Composition 1 > Composition 2 > Composition 3. Since the same amount of cream was treated for the SDS-PAGE experiment as described in "Materials and methods" part, it is natural that the total intensity of each emulsion sample ranked in the same order. High-mass materials were found at the top of the gel of all the emulsion samples, suggesting the protein aggregation induced by the severe heat treatment for sterilization.

There was a clear difference in the composition of protein bands between the control and the other emulsions. For main components of milk protein, α- and β-caseins and α-lactalbumin disappeared in the patterns of Compositions 1 and 2, whereas these protein bands were clearly shown in the control emulsion. κ-Casein and β-lactoglobulin remained as adsorbed proteins in Compositions 1 and 2 emulsions. These results, on the displacement of milk proteins by emulsifiers, correspond to those of previous studies. Mackie, Gunning, Ridout, Wilde & Morris (2001) reported that a nonionic surfactant preferentially displaces β-casein before β-lactoglobulin from the air/water interface. It was also shown that β-casein was more easily displaced than β-lactoglobulin from the surface of oil droplets by oil soluble emulsifiers (Cornec, Wilde, Gunning, Mackie, Husband, Parker & Clark, 1998). In our case, the emulsifiers such as DP-95, BS-20 and S-550,
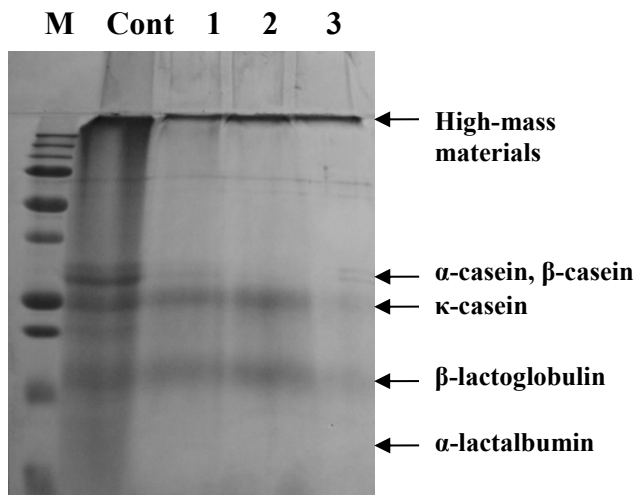
M   Cont   1   2   3

← High-mass materials

← α-casein, β-casein
← κ-casein

← β-lactoglobulin

← α-lactalbumin

Figure 1-6. Compositions of adsorbed proteins at the oil droplet surface in emulsions

The adsorbed proteins at the oil droplet surface in emulsions were collected according to the procedure of Figure 1-4, and their composition was analyzed by SDS-PAGE. M shows a molecular weight marker consisting of proteins with the following molecular weight; 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa from the top. The adsorbed proteins were identified by comparing their mobility with that of purified components of milk protein fractions.

Cont: Control (very stable emulsion),
1: Composition 1 (stable emulsion),
2: Composition 2 (stable emulsion),
3: Composition 3 (unstable emulsion).

which were used in the preparation for Compositions 1 and 2 emulsions, should have the ability to displace α and β-casein, but could not act on the adsorbed layer of κ-casein and β-lactoglobulin. In the presence of Composition 3 emulsion, however, the band of κ-casein and β-lactoglobulin turned out to be faint, suggesting the displacement of these proteins to a larger extent. The use of more surface active emulsifier with high HLB such as P-1670 possibly enabled most of κ-casein and β-lactoglobulin to be desorbed from the oil droplets surface.

From the results of SDS-PAGE of adsorbed proteins, we can speculate that there is a close relation between the composition of the adsorbed proteins and the stability of the emulsions. The adsorbed protein fraction of control emulsion included all the major components of milk proteins such as α-, β-, κ-casein β-lactoglobulin and α-lactalbumin. However, for Compositions 1 and 2 emulsions, which were less stable than the control emulsions, but still can maintain the dispersion

of oil droplets after 3 months storage, only κ-casein and β-lactoglobulin were present. This means that κ-casein and β-lactoglobulin are essential for preventing oil droplets from aggregating and coalescing, although α- and β-casein may enhance the emulsion stability. κ-Casein has a carbohydrate moiety in the C-terminal region, which provides casein micelles with colloidal stability by means of steric and electrostatic repulsion (Modler, 1985). For Compositions l and 2 emulsions, adsorbed κ-casein may contribute to the stability of emulsions by the action of its C-terminal region. On the other hand, it is known that β-lactoglobulin forms a more viscoelastic film at the interface than α- and β-caseins (Murray & Dickinson, 1996). In addition to the steric hindrance effect, β-lactoglobulin may contribute to the emulsion stability via the formation of a viscoelastic adsorbed layer, which is resistant to the coalescence of oil droplets.

4. CONCLUSION

The purpose of this study was to find the factors affecting the stability of milk-based emulsions which are used for the production of canned coffee. The data from the study suggests that the amount and composition of adsorbed proteins at the oil droplet surface plays an important role in the emulsion stability. This finding should be useful for predicting the long-term stability of milk-based emulsions and designing the best formulation for emulsion products such as canned coffee and tea.

REFERENCES

Bartlett, G.R. (1959). Phosphorous assay in column chromatography. *The Journal of Biological Chemistry* 234, 466-468.

Bligh, E.G. & Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* 37, 911-917.

Bos, M.A. & van Vliet, T. (2001). Interfacial rheological properties of adsorbed protein layers and surfactants: a review. *Advances in Colloid and Interface Science* 91, 437–471.

Chanamai, R. & McClements, D.J. (2000). Creaming stability of flocculated monodisperse oil-in-water emulsions. *Journal of Colloid and Interface Science* 225, 214–218.

Cornec, M., Wilde, P.J., Gunning, P.A., Mackie, A.R., Husband, F.A., Parker, M.L. & Clark, D.C., (1998). Emulsion stability as affected by competitive adsorption between an oil-soluble emulsifier and milk proteins at the interface. *Journal of Food Science* 63, 39-43.

Courthaudon, J.L., Dickinson, E. & Dalgleish, D.G. (1991). Competitive adsorption of β-casein and nonionic surfactants in oil-in-water emulsions. *Journal of Colloid and Interface Science* 145, 390-395.

De Feijter, J.A., Benjamins, J. & Tamboer, M. (1987). Adsorption displacement of proteins by surfactants in oil-in-water emulsions. *Colloids and Surfaces* 27, 243-266.

Dickinson, E. (2001). Milk protein interfacial layers and the relationship to emulsion stability and rheology. *Colloids and Surfaces B: Biointerfaces* 20, 197-210.

Dickinson, E., Euston, S.R. & Woskett, C.M. (1990). Competitive adsorption of food macromolecules and surfactants at the oil-water interface. *Progress in Colloid and Polymer Science* 82, 65-75.

Dickinson, E. & Tanai, S. (1992). Protein displacement from the emulsion droplet surface by oil-soluble and water-soluble surfactants. *Journal of Agricultural and Food Chemistry*

40, 179-183.

Euston, S. E., Singh, H., Munro, P. A. & Dalgleish, D. G. (1995). Competitive adsorption between sodium caseinate and oil-soluble and water-soluble surfactants in oil-in-water emulsions. *Journal of Food Science* 60, 1124-1131.

Fang, Y. & Dalgleish, D.G. (1993). Casein adsorption on the surfaces of oil-in-water emulsions modified by lecithin. *Colloids and Surfaces B: Biointerfaces* 1, 357-364.

Friberg, S.E., Quencer, L.G. & Hilton, M.L. (1996). Theory of Emulsions. In: *Pharmaceutical Dosage Forms: Disperse Systems*, second ed., volume.1. Marcel Dekker, New York.

Kabara, J.J., Swieczkowski, D.M., Conley, A.J. & Truant, J.P. (1972). Fatty acids and derivatives as antimicrobial agents. *Antimicrobial Agents Chemotherapy* 2, 23-28.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* 193, 265-275.

Mackie, A.R., Gunning, A.P., Ridout, M.J., Wilde, P.J. & Morris, V.J. (2001). Orogenic displacement in mixed β-lactoglobulin/β-casein films at the air/water interface. *Langmuir* 17, 6593-6598.

Matsumiya, K., Takahashi, Y., Nakanishi, K., Dotsu, N. & Matsumura, Y. (2007). Destabilization of O/W emulsions by the diglycerol ester. In: Proceedings of the 98th AOCS Annual Meeting & Expo, Quebec, Canada.

Matsumura, Y. (2005). Effects of fat crystallization on emulsion stability. *Oleoscience* 5, 13-19.

McClements, D.J. (2005a). *Food Emulsions: Principles, Practice, and Techniques*, second ed. CRC Press, Boca Raton. pp. 515-526.

McClements, D.J. (2005b). *Food Emulsions: Principles, Practice, and Techniques*, second ed. CRC Press, Boca Raton. pp. 1-26.

Modler, H.W. (1985). Functional properties of nonfat dairy ingredients - a review. Modification of

products containing casein. *Journal of Dairy Science* 68, 2195-2205.

Murray, B.S. and Dickinson, E. (1996). Interfacial rheology and the dynamic properties of adsorbed films of food proteins and surfactants. *Food Science and Technology International* 2, 131-145.

Mutoh, T., Kubouchi, H., Noda, A., Shiinoki, Y. & Matsumura, Y. (2007). Effect of oil-soluble emulsifiers on solidification of thermally treated creams. *International Dairy Journal* 17, 24-28.

Shibasaki, I. (1979). Antimicrobial activity of fatty acids and their esters (in Japanese). *Hakkokogaku Kaishi* 57, 164-176.

Tcholakova, S., Denkov, N.D., Ivanov, I.B. & Campbell, B. (2002). Coalescence in β-lactoglobulin-stabilized emulsions: effects of protein adsorption and drop size. *Langmuir* 18, 8960-8971.

Wade, T. & Beattie, J. K. (1997). Electroacoustic determination of size and zeta potential of fat globules in milk and cream emulsions. *Colloids and Surfaces B: Biointerfaces* 10, 73-85.

Walstra, P., Wouters, J.T.M. & Geurts, T.J. (2006). *Dairy Science and Technology*, second ed. CRC press, Boca Raton. pp. 17-108.

Wilde, P., Mackie, A., Husband, F., Gunning, P. & Morris, V. (2004). Proteins and emulsifiers at liquid interfaces. *Advances in Colloid and Interface Science* 108-109, 63-71.

Chapter 2:

Destabilization of protein-based emulsions by diglycerol esters of fatty acids -The importance of chain length similarity between dispersed oil molecules and fatty acid residues of the emulsifier

1. INTRODUCTION

Food products undergo various degradations during their long-term shelf-life; for example, undesirable changes of colors, off-flavors, or microorganism contaminations. For canned foods, food manufactures particularly have to pay attention to bacterial poisoning caused by *Bacillus* spp. and *Clostridium* spp. which form heat-resistant spores (Parker, 2003; Engelkirk & Engelkirk, 2007). The sporeformers are generally prevented from growing by using emulsifiers with bacteriostatic effects especially in emulsion-type foods. Kabara, Swieczkowski, Conley & Truant (1972) and Kato & Shibasaki (1975) reported the bacteriostatic effects of fatty acids esters. Despite the name "emulsifiers", bacteriostatic emulsifiers often promote destabilization of protein-based emulsions. Diglycerol esters of fatty acids are well-known to food manufacturers as bacteriostatic emulsifiers. These emulsifiers are categorized to polyglycerol esters of fatty acids, in which the polymerization degree of glycerol and the kind of fatty acids can be changed. Yamazaki, Yamamoto, Kawai & Inoue (2004) reported effects of diglycerol fatty acid esters with different carbon chain lengths with/without essential oil constituents on a bacterium. Diglycerol esters of fatty acids are surface active and reduce interfacial tension between oil phase and aqueous phase, but, under conditions, they can contribute to severe coalescence between oil droplets in otherwise stable emulsions (Holstborg, Pedersen, Krog & Olesen, 1999).

Coalescence is the process where two or more droplets merge together to form a larger droplet. It is mainly affected by interfacial structures between oil and water, environmental

conditions around oil droplets and processing of emulsions (McClements, 2005). Protein emulsifiers adsorbed to the oil droplet surfaces effectively retard droplet coalescence by forming protective membranes around the droplets (Dickinson, 1992b; McClements, 2004). Processing such as application of shear forces, freeze-thaw, drying may promote coalescence due to the increase of droplet-droplet contacts (McClements, 2005). In the chilled state, fat crystallization promotes partial coalescence (Rousseau, 2000) which causes the consistency of emulsions (Dicknson & McClements, 1996).

In the previous studies, it was reported that bacteriostatic emulsifiers could displace proteins from the oils droplet surface (Holstborg et al., 1999; Chen & Dickinson, 1999; Matsumiya, Takahashi, Inoue & Matsumura, 2010). The protein displacement by emulsifiers could be an important factor, but it does not always lead to the destabilization of emulsions (Matsumiya, Takahashi, Nakanishi, Dotsu & Matsumura, 2007). The authors also found the ability of diglycerol ester of mono-oleate, representative of the emulsifiers under study, to migrate from oil phase to aqueous phase and vice versa (Matsumiya et al., 2007).

Soybean oil was used in our previous studies (Matsumiya et al., 2007). To investigate the phenomena occurred in emulsions systems more precisely, simple oil phase such as hydrocarbon is very often used for the experiments (Small, 1986a). The authors also used several hydrocarbons in the preliminary experiment, and found that the emulsion destabilization caused by diglycerol esters of mono-oleic acid varied with the oil phase types. This indicates the importance of structural feature of oil molecules in the destabilization process of emulsions by the bacteriostatic emulsifier.

In this context, the author examined effects of oil-phase type on the emulsion destabilization by the bacteriostatic emulsifier in the present study. In addition to the difference of oil type (triacylglycerol versus hyrocarbon), the importance of oil chain length of hydrocarbon was also tested. Another important factor is the difference in fatty acid residue linked to diglycerol esters.

For air/water interfaces, interfacial and foaming characteristics of diglycerol esters-milk proteins mixed solutions were examined in the previous studies, and then confirmed that they depend on the carbon chain length of the emulsifiers (Álvarez & Rodríguez Patino, 2006; Álvarez, Ruíz-Henestrosa, Sánchez, & Rodríguez Patino, 2008). Therefore, concerning emulsions rather than interfaces, the author investigated the effects of the combination of five hydrocarbons in the oil phase and five emulsifiers (five diglycerols esterified with different mono-fatty acid). The purpose of this work is to study how the structural relationship between oil molecules and fatty acid residues of emulsifiers affects the destabiliztion of protein-based emulsions.

2. MATERIALS AND METHODS

2.1 Materials

Deionized water was used for the preparation of all solutions. Corn oil was obtained from Nacalai Tesuque, Inc. (Kyoto, Japan). Soybean oil and four hydrocarbons ($C_{10}H_{22}$, hereafter abbreviated to [C10], $C_{12}H_{26}$ [C12], $C_{14}H_{30}$ [C14], $C_{16}H_{34}$ [C16]; purity, > 95 %) were supplied by Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Octadecane ($C_{18}H_{38}$ [C18]; purity, > 95 %) was supplied by Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Diglycerol esters of different mono-saturated or unsaturated fatty acids [DF] with bacteriostatic effects (diglycerol esters of mono-lauric acid, hereafter abbreviated to [DL], mono-myristic acid [DM], mono-palmitic acid [DP], mono-stearic acid [DS] and mono-oleic acid [DO]; purity, > 95 %) and sodium caseinate [Na-CN] were manufactured by Riken Vitamin Co., Ltd (Osaka, Japan). All other general chemicals used were of analytical grade purchased from Nacalai Tesuque, Inc. (Kyoto, Japan).

2.2 Emulsion preparation

Protein solutions were prepared by dissolving sodium caseinate into pH 7.0, 40 mM phosphate buffer. Stock emulsions were prepared by homogenizing oils (food oils or hydrocarbons) and the protein solution with a high-speed blender at 60 ºC at 19,300 rpm for 3 minutes (Physcotron,

NS-51, Microtec Co., Ltd., Chiba, Japan) followed by ultrasonic treatment using an ultrasonic generator for 5 minutes (Nissei, Tokyo, Japan). The contents of oil, protein and buffer in stock emulsions were 10.0, 0.4 and 89.6 wt % respectively. Emulsifier solutions were prepared by dispersing DF into water using a high-speed blender at 60 ºC at 19,300 rpm for 3 minutes (Physcotron, NS-51, Microtec Co., Ltd., Chiba, Japan). Sample emulsions for stability tests were prepared by mixing emulsifier solution (0.4 wt% DF in deionized water) with the stock emulsion at the volume ratio of 1:1. Emulsions without DF (Control emulsions) were made by simply mixing buffer and stock emulsions in Section 3.2. The prepared emulsions were stored at 35 ºC (Section 3.1) or 60 ºC (Section 3.2).

2.3 Tensiometry

The interfacial properties of DO and sodium caseinate were examined by using Wilhelmy Plate method (Automatic Surface Tensiometer, CBVP-A3, Kyowa Interface Science Co., Ltd., Japan) at 35 °C. The emulsifier and protein were dissolved in aqueous phase to give 0.1 wt% to the total weight of aqueous phase, respectively.

2.4 Evaluation of proteins adsorbed to the oil droplet surface

Small aliquots of sample emulsion were gently filtered by using a syringe driven membrane unit (0.2 μm pore) to remove oil droplets and collect aqueous phase. Protein content of the aqueous phase was determined by using the Lowry's method and sodium caseinate as a standard (Lowry, Rowebrough, Farr & Randall, 1951). The relative amount of proteins adsorbed to the oil droplet surface was calculated from the protein content of the aqueous phase.

2.5 Periodical observations and measurements

2.5.1 Visual observations

Emulsion destabilization such as creaming, coalescence or phase separation caused by the diglycerol esters of fatty acids was visually observed. These destabilization phenomena were

recorded by taking pictures from 0 min (just after adding emulsifiers) to 60 min.

2.5.2 Particle size analyses

The particle size distribution of prepared emulsions was measured using a laser-diffraction particle size analyzer (SALD-2200, Shimadzu Corporation, Kyoto, Japan), which measures the angular intensity of laser light scattering from a diluted emulsion. Sample emulsions were diluted to an appropriate concentration using deionized water in order to avoid multiple scattering. A refractive index of 1.45-0.50 i was used to calculate the particle size distribution on the Mie theory. The particle size was reported as the volume-weighted average diameter, $d_{4,3}$ ($=\sum f_i d_i^4 / \sum f_i d_i^3$, where f_i is the frequency of particles with diameter d_i).

2.6 Statistical analysis

All experiments were conducted in more than triplicate with freshly prepared sample emulsions. Effects of storage-term and oil-phase type on mean particle diameter were assessed using a two-way repeated-measures ANOVA, with storage-term (Control/ 0 min/ 30 min/ 60 min) as one factor and oil-phase type (C16/ C18/ Soybean oil/ Corn oil) as the other, or with oil-phase type (C10/ C12/ C14/ C16/ C18) as the other. In section 3.2, after 60 min storage, ANOVAs were followed by comparisons between mean particle diameters in various emulsions prepared with the five hydrocarbons using Tukey's comparison method to report significantly destabilized emulsions in terms of microscopically-observed coalescence.

Effects of emulsifier type and oil-phase type on interfacial tension between oil and aqueous phase were also assessed using a two-way repeated-measures ANOVA, with emulsifier type (DO and/or Na-CN) as one factor and oil-phase type (C16/ C18/ Soybean oil/ Corn oil) as the other. The level for statistical significance was set $p < 0.05$ for all statistical analyses. Two-way ANOVA and Tukey's comparison methods were performed using Microsoft Excel 2003 for Windows and Ekuseru-Toukei 2006 software (Social Survey Research Information Co., Ltd.; Shinjuku, Tokyo).

3. RESULTS AND DISCUSSION

3.1 Effects of oil-phase type on the emulsion destabilization

It is practically known by the emulsifier manufacturer that DO has the strongest destabilizing effect on protein-based emulsions prepared with food oils among the five emulsifiers. Kumar, T. N., Sastry, Y. S. R. & Lakshminarayana, G. (1989) reported the same phenomenon in their research. First, the author examined the effects of oil-phase type on the emulsion destabilization by DO. Experiments in this section were performed at 35 ℃ to avoid crystallization of any oil used in this research. Crystallization of oil phase affects partial coalescence. The melting point of soybean oil, corn oil, C10, C12, C14, C16 and C18 is approximately -22, -12, -30, -10, 6, 18 and 28 °C, respectively (Small, 1986c; O'Brien, 2003).

Fig. 2-1a shows the appearance of sample emulsions prepared with four types of oil, that is, C16, C18, Soybean oil and Corn oil. No creaming was observed in all the emulsions just after the addition of DO (0 min), while creaming of C16 emulsion was observed after 60 min storage at 35 ℃. Creaming can be caused by flocculation due to the increase of efficient hydrodynamic radius (Dickinson, 1992a; Bremer, Bijsterbosch, Walstra, & van Vliet, 1993) or coalescence according to Stokes' law. The difference of oil densities between the food-grade oils and the hydrocarbons (about 0.2 g/ml) should be small and cannot affect creaming. The author measured the mean particle diameter of oil droplets. The particle size of C16 emulsion was the largest of all the emulsions after 60 min (two-way ANOVA, $p < 0.05$) (Fig. 2-1b), suggesting that coalescence of oil droplets occurred in C16 emulsion. In order to confirm this phenomenon, the author we observed the destabilizing behavior of C16 emulsion by light microscopy, and the progress of coalescence was observed only in C16 emulsion (Fig. 2-1b). These results suggest that creaming in C16 emulsion can be attributed to the oil droplet coalescence.
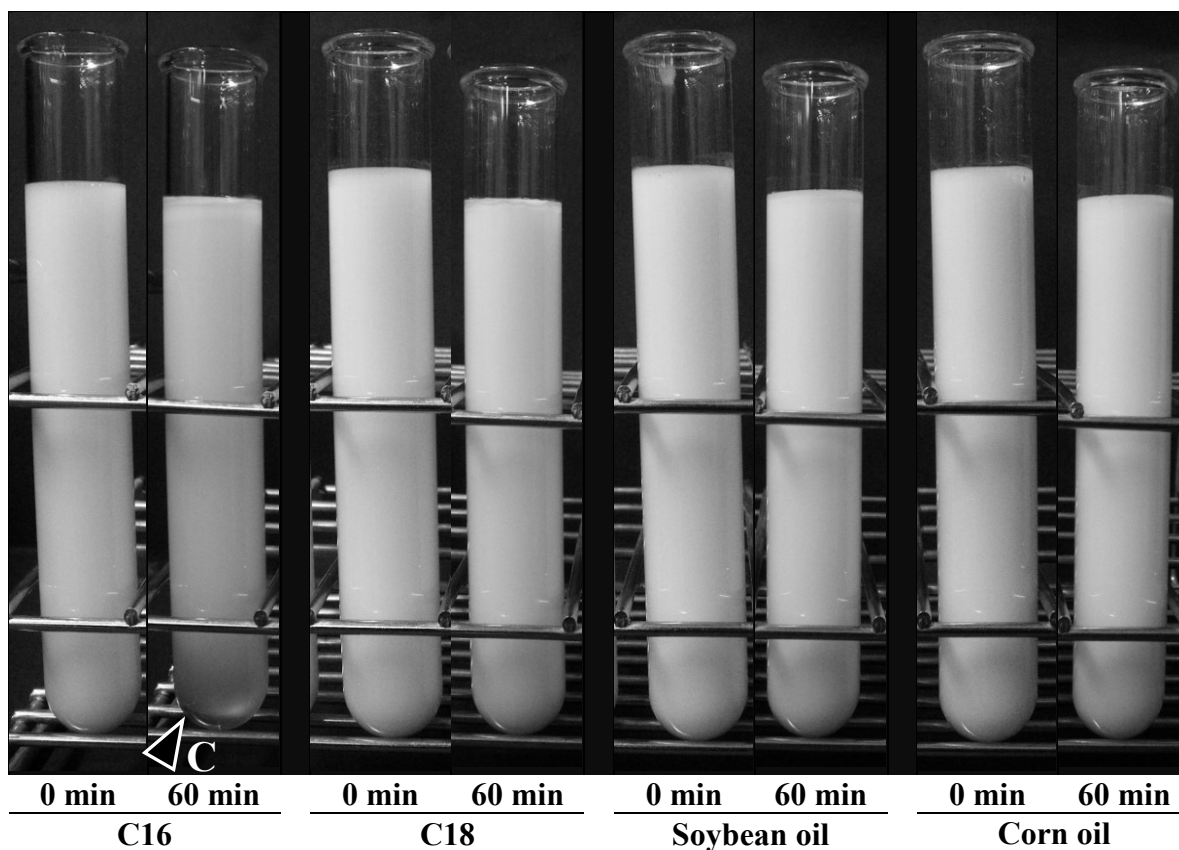
Fig. 2-1a. Effects of oil-phase type on the appearance of emulsions (food oil versus hydrocarbon) Sample emulsions were stored at 35 ºC and recorded at 0 min after and 60 min after the preparation. DO was used for the preparation of sample emulsions. "C" in the picture indicates that the creaming was observed for the emulsion.

To give an insight into the physical state of oil droplet surfaces, the author measured interfacial tension at the oil-water interface by Wilhelmy's plate method in plane interface system (Fig. 2). Protein adsorption and emulsifier adsorption to the oil-water interface can be predicted by measuring the interfacial tension (Krog, N. 1984; Holstborg *et al.*, 1999). Both emulsifier and oil-phase type significantly affected the interfacial tension (two-way ANOVA, $p < 0.05$). The interfacial tension between oil and water in the presence of Na-CN was 16.8, 9.6, 7.3 or 4.6 mN/m for C16, C18, Soybean oil and Corn oil cases, respectively. The interfacial tension in the presence of DO was close to 0, close to 0, 7.3 or 4.5 mN/m, respectively. Meanwhile, the interfacial tension in the presence of both Na-CN and DO was close to 0, close to 0, 7.6 or 4.6 mN/m, respectively.
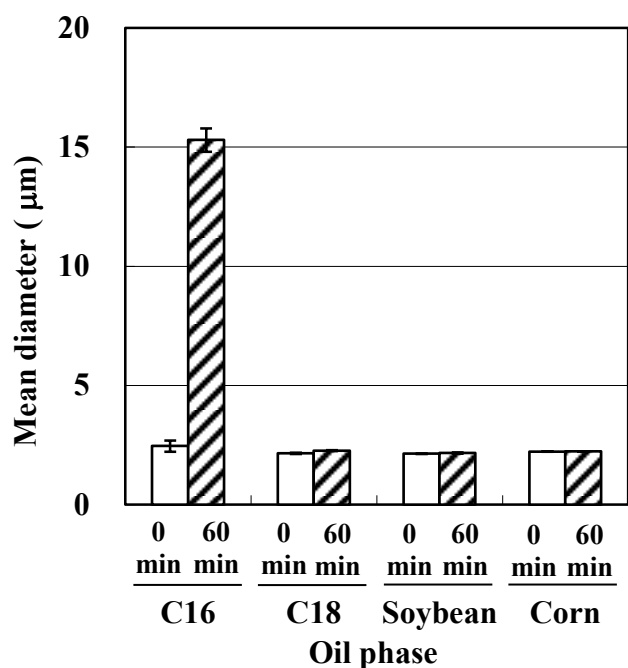
Figure 2-1b. Effects of oil-phase type on the mean particle diameter of oil droplets (food oil versus hydrocarbon)

Sample emulsions were stored at 35 ℃. Particle size analyses were performed 0 min after and 60 min after the preparation of sample emulsions. Mean particle diameter was represented by $d_{4,3}$. C16 emulsion was observed by a light microscopy 0 min and 60 min after the addition of DO.
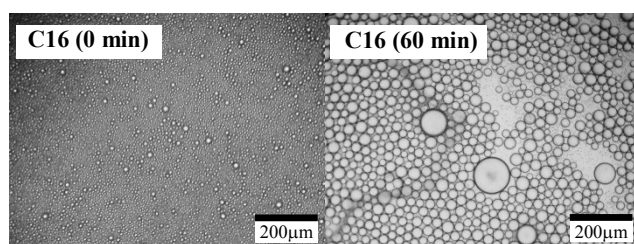
From these results, proteins adsorbed to the plane oil-water interface of C16 and C18 oils seemed to be completely displaced, while most of proteins adsorbed to interface of soybean and corn oils seemed to remain at the interface after the addition of DO. In addition to this experiment, we performed evaluation of the relative amount of proteins adsorbed to the oil droplet surface after the addition of DO in emulsion system. Resultantly, it was revealed that negligible amount of proteins (1.9 ± 1.7 wt% of the added proteins for C16 emulsion and 0.9 ± 2.7 wt% for C18 emulsion, respectively) remained on the oil droplet surface in the hydrocarbon emulsions, whilst more amount of proteins (15.0 ± 6.0 wt% for soybean oil emulsion and 9.9 ± 2.5 wt% for corn oil emulsion, respectively) remained on the oil droplet surface in the food-oil emulsions. These data correspond to the result obtained in the plane interface system (Fig. 2-2).
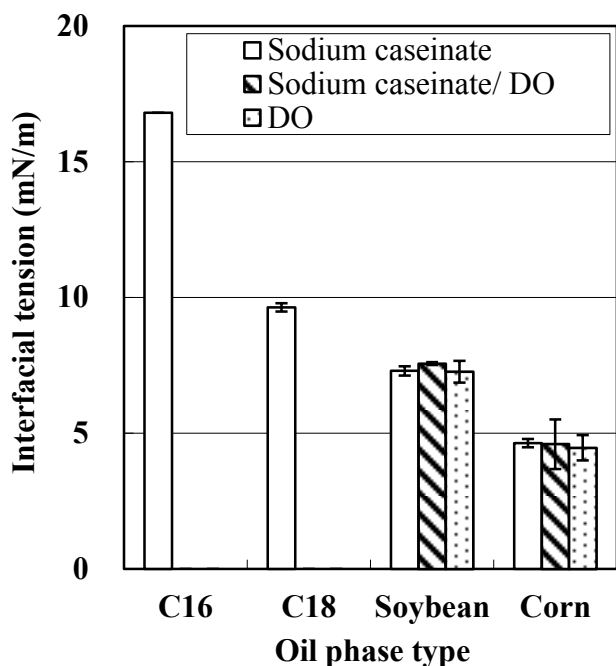
Figure 2-2. Interfacial tension of DO and/or Na-CN in aqueous phase against oil (C16, C18, Soybean oil and Corn oil) at 35 °C measured by the Wilhelmy's plate method.

These results indicate that the complete displacement of proteins from the hydrocarbon oil droplet surfaces by DO does not necessarily cause the destabilization of emulsions. To confirm the destabilization of other hydrocarbon oil emulsions by DO, the author again performed periodical observations and particle size measurements of the emulsions prepared with various hydrocarbons, in addition to C16 and C18. Just after and 30 min after the preparation of sample emulsions, no creaming was observed in all the emulsions (Fig. 2-3a). Meanwhile, creaming of C16 emulsion and slight creaming of C12 and C14 emulsions were observed 60 min after the preparation of samples.

The time-dependent measurement of oil droplet size revealed that emulsion destabilization occurred in C12, C14 and C16 emulsion, corresponding to the result of visual observations (Fig. 2-3b). It should be noted that a steep increase of oil droplet size was observed only in C16 emulsion, indicating that DO particularly acts on the C16 oil droplet. These phenomena might be closely related to the structural similarity, that is, the carbon chain length similarity between oil molecules and fatty acid residue of the emulsifier, DO. Calculated apparent carbon chain length of five hydrocarbons and fatty acid residues including oleic acid residue, as a part of DF, are shown in

Table 2-1 (Small, 1986b; Morrison & Boyd, 1992). Effective carbon chain length of oleic acid is similar to that of C16 rather than that of C18. The carbon chain length of other hydrocarbons, C10, C12 and C14 are obviously shorter than that of oleic acid residue. Based on the comparison between the calculated data in Table 2-1 and the results of Fig. 2-3, DO selectively destabilized C16 oil droplets to a great extent possibly by the similarity between the chain length of oleic acid (18.75 $\overset{\circ}{A}$) and that of C16 (19.05 $\overset{\circ}{A}$). The author carried out the more systematic approach, that is, the author investigated the effects of the combination of hydrocarbon and DF shown in Table 2-1 on the emulsion stability in the next section.

3.2 Effects of chain length similarity between dispersed oil molecules and fatty acid residues of the emulsifiers on the emulsion destabilization

The effects of the combination of five hydrocarbons in oil phase and five emulsifiers on the emulsion destabilization were investigated. Experiments were performed at 60 ℃ to avoid the crystallization of hydrocarbons and to avoid the solidification of emulsifiers. Visual observations were periodically performed just after, 30 min after and 60 min after the preparation of sample emulsions. Because immediate destabilization was observed in particle size analyses of emulsions just after the preparation of sample emulsions in some cases, the author prepared sample emulsions with no emulsifier as a control and confirmed that the immediate destabilization was not caused only by the mechanical force of gently mixing.

In this section, phase separation was observed as well as creaming and microscopically-observed micro-ordered coalescence. To avoid ambiguous judgments of emulsion stability, the author established the following criteria. The author categorized destabilizations into three types of destabilization, that is, creaming, phase separation (large-scale coalescence and clear appearance of oil at the top) and microscopically-observed micro-ordered coalescence. For creaming, emulsion destabilizations were distinguished into no creaming, slight creaming (less than
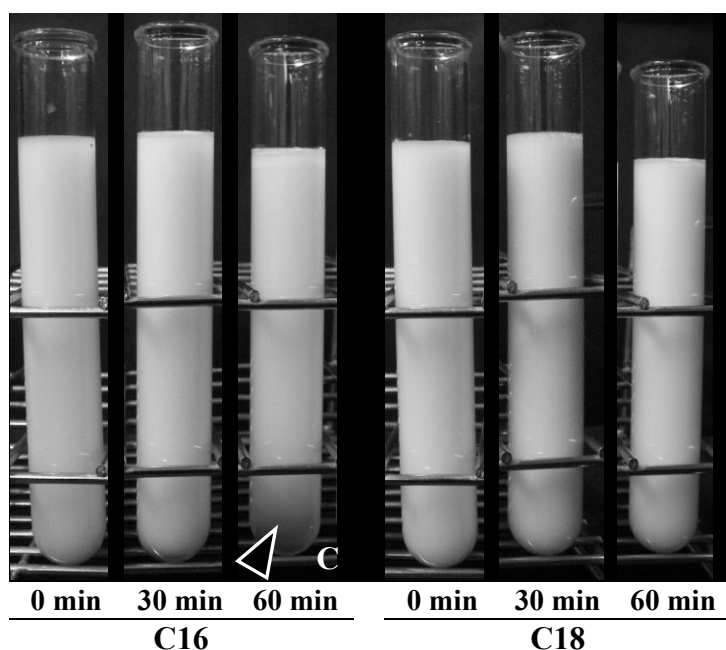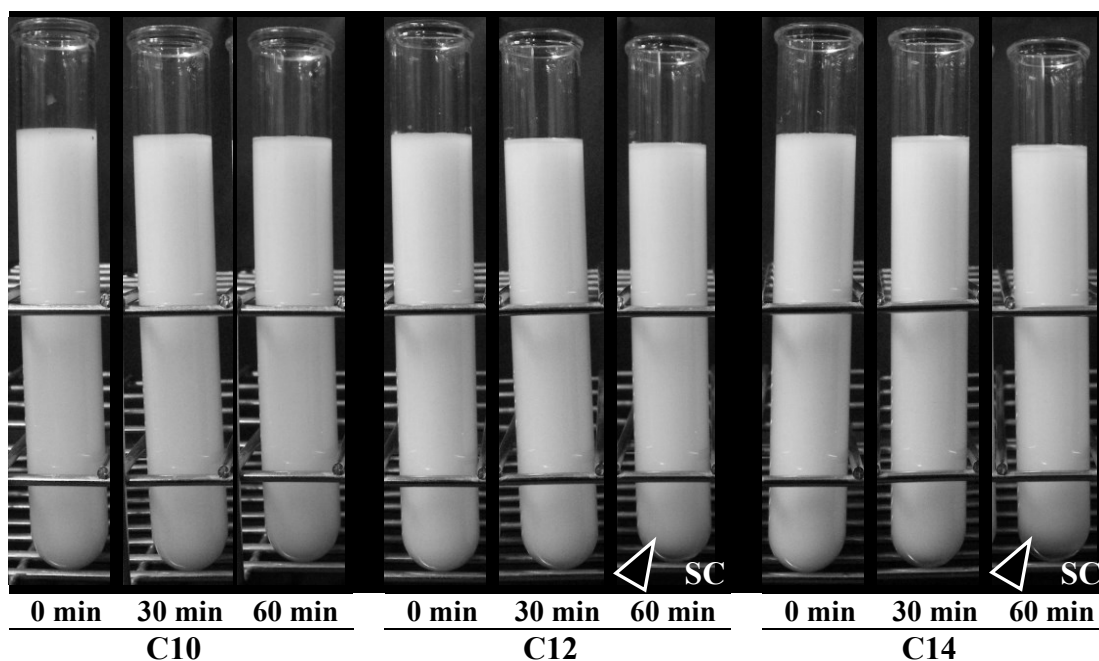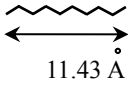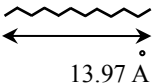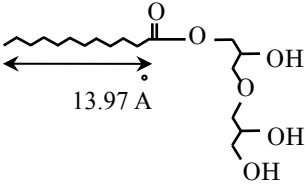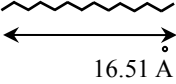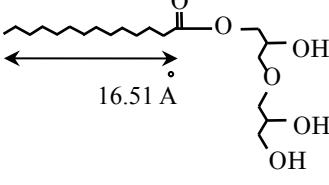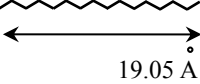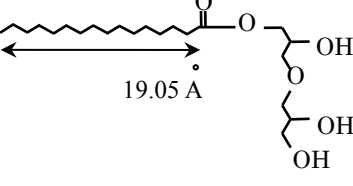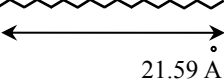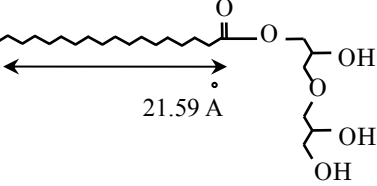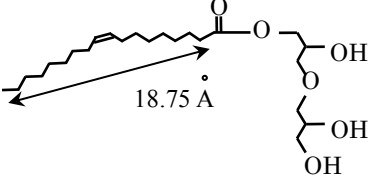
Figure 2-3a. Effects of oil-phase type on the appearance of sample emulsions (five hydrocarbons)

Sample emulsions were stored at 35 ºC and recorded at 0 min after, 30 min after and 60 min after the preparation. DO was used for the preparation of sample emulsions. "SC" and "C" in the picture indicate that the slight creaming or creaming was observed, respectively.

half) and creaming (more than half). For phase separation, the author judged whether phase separation was observed or not. For microscopically-observed coalescence, the author defined significantly destabilized emulsions in droplet size as unstable emulsions by Tukey's comparison method after two-way ANOVA.

Fig. 2-4a (i) shows the effects of DL (C12:0) on the appearance of emulsions of five hydrocarbons, C10, C12, C14, C16 and C18. C10 and C16 emulsion were particularly destabilized

Table 2-1. Molecular structure and calculated carbon chain length of hydrocarbons and DF

Hydrocarbons	Diglycerol esters of fatty acid

C10 11.43 Å

C12 13.97 Å   DL 13.97 Å

C14 16.51 Å   DM 16.51 Å

C16 19.05 Å   DP 19.05 Å

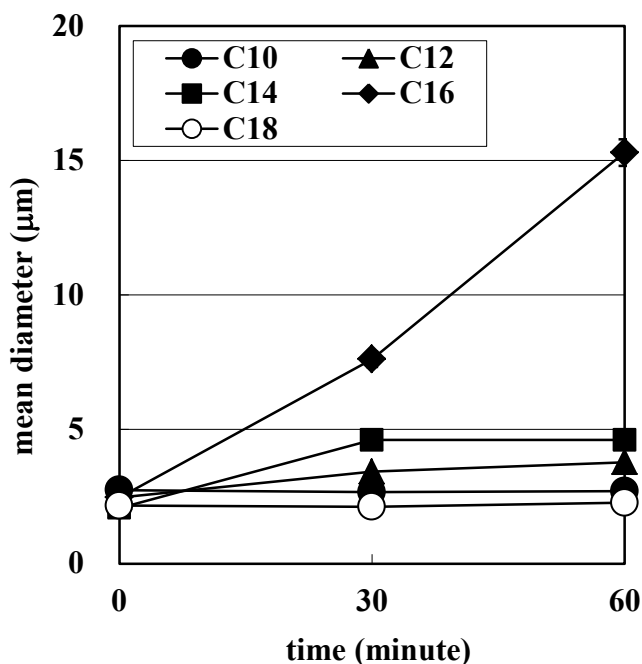C18 21.59 Å   DS 21.59 Å

DO 18.75 Å

Figure 2-3b. Effects of oil-phase type on the mean particle diameter of oil droplets (five hydrocarbons)

Sample emulsions were stored at 35 ºC. Particle size analyses were performed 0 min after, 30 min after and 60 min after the preparation of sample emulsions. Mean particle diameter was represented by $d_{4,3}$.

and the clear separation of cream layer and serum phase was observed 60 min after the preparation of sample emulsions. However, no significant change of oil droplet particle size was periodically observed for all of the hydrocarbon emulsions including C10 and C16 one (two-way ANOVA) (Fig. 2-4b (i)). The creaming of C10 and C16 emulsion can be attributed to the oil droplet flocculation which accelerates the rate of gravitational separation (Robins, 2000). These results are briefly summarized in Table 2-2.

Effects of DM (C14:0) on the emulsions were examined in the same manner. For the creaming, C10, C12 and C14 emulsion were destabilized and C16 emulsion was slightly destabilized (Fig. 2-4a (ii)). C10, C12 and C14 emulsion separated into the distinct two phases. Mean particle diameters of C10, C12, C14 and C16 emulsion periodically increased and they varied with the oil phase type used in this work (two-way ANOVA, $p < 0.05$). The mean diameter of oil droplets for C10, C12 and C14 was significantly larger than that for C16 and C18 after 60 min storage (Tukey's method, $p < 0.05$) (Fig 2-4b (ii)). C10 emulsion with DM was extremely unstable, and the large mean diameter was observed just after the preparation of sample emulsions.
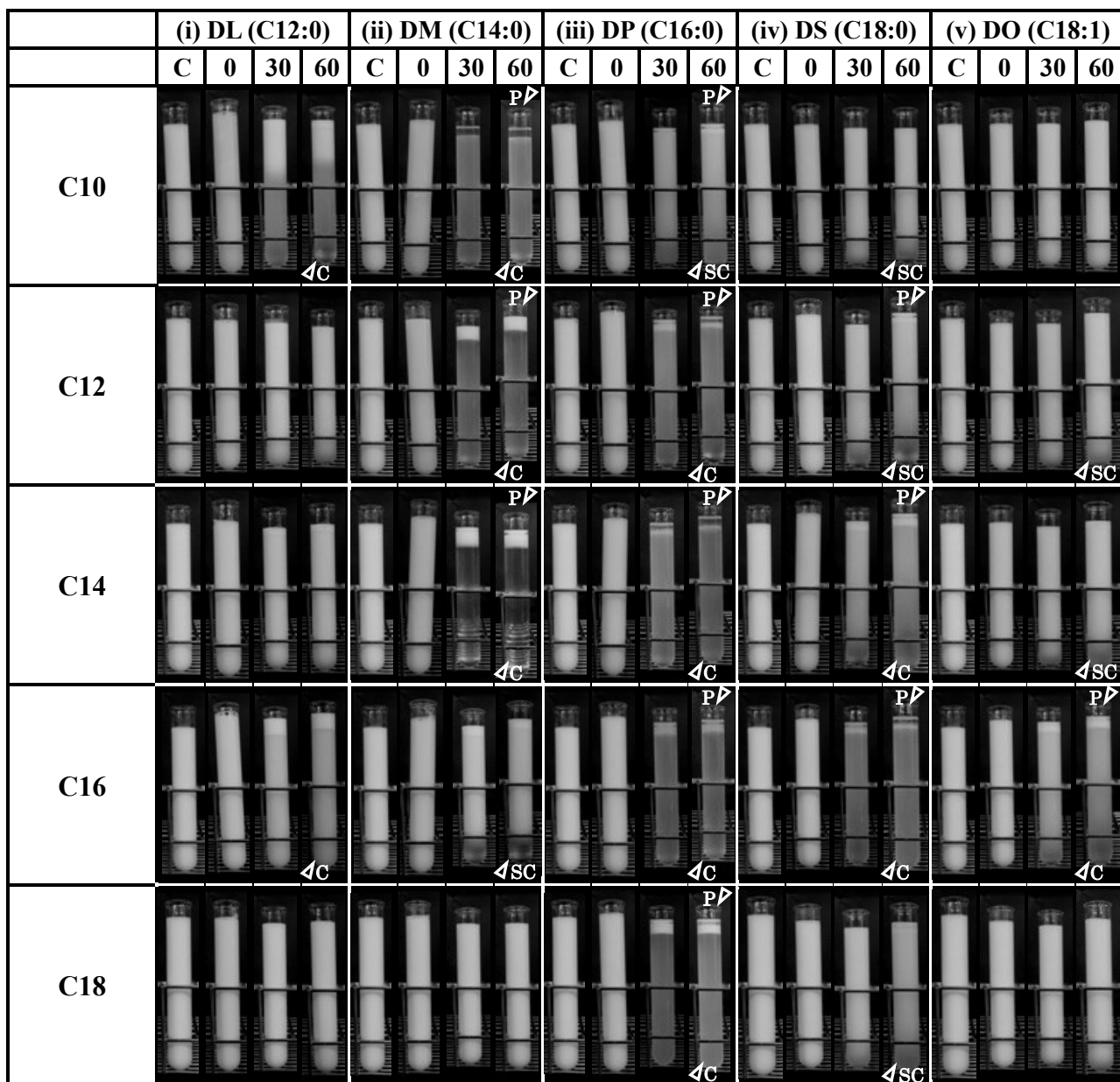
Figure 2-4a. Effects of the combination of five hydrocarbons and five DF on the appearance of sample emulsions

Sample emulsions were stored at 60 ℃ and recorded at 0 min after, 30 min after and 60 min after the preparation. Control indicates the emulsion with no emulsifier 0 min after the preparation. (Control, 0 min, 30 min and 60 min are represented as C, 0, 30 and 60 in the second line, respectively.) "SC" and "C" in the picture indicate that the slight creaming or creaming was observed, respectively. "P" means that phase separation was observed in the emulsion.
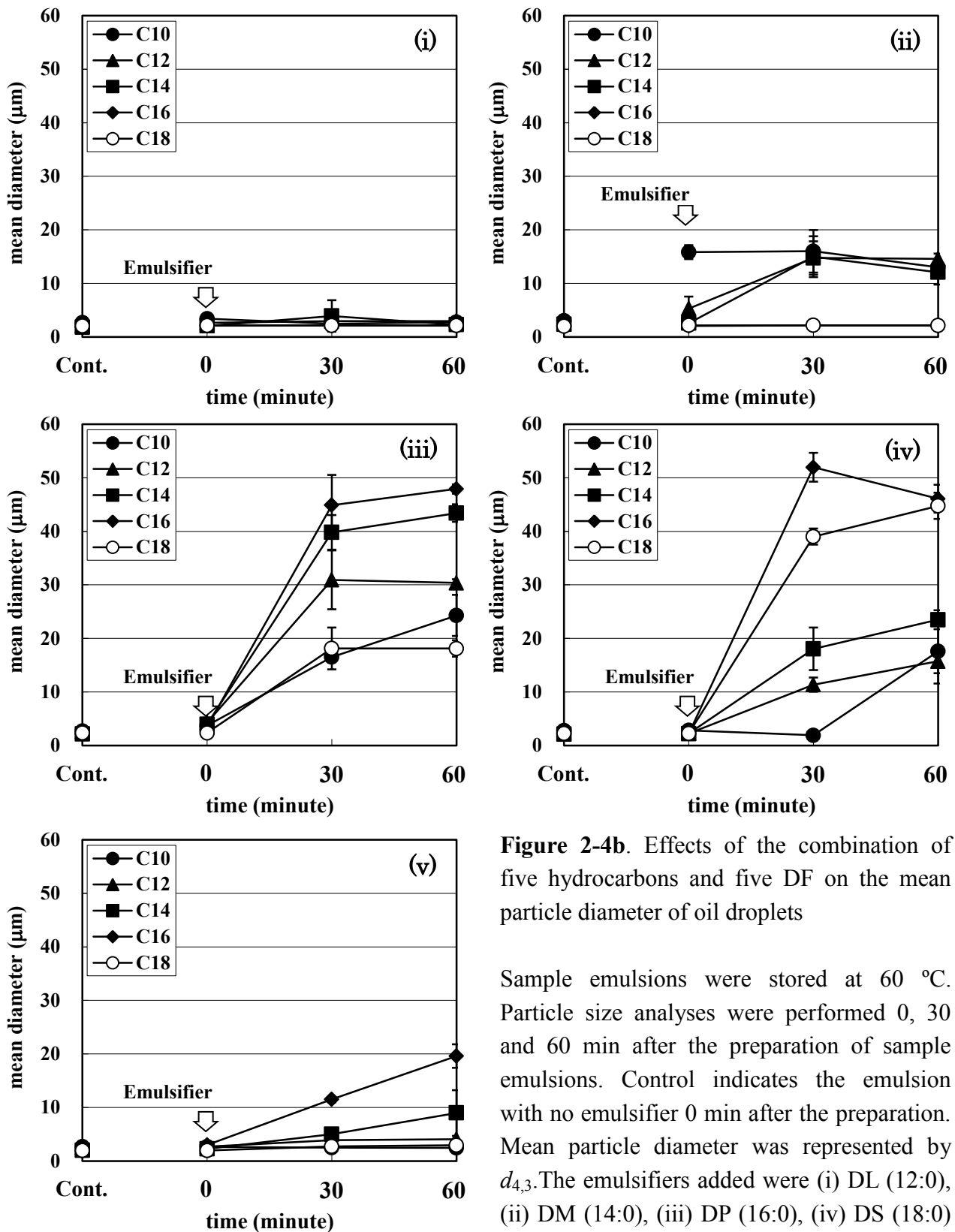
Figure 2-4b. Effects of the combination of five hydrocarbons and five DF on the mean particle diameter of oil droplets

Sample emulsions were stored at 60 ℃. Particle size analyses were performed 0, 30 and 60 min after the preparation of sample emulsions. Control indicates the emulsion with no emulsifier 0 min after the preparation. Mean particle diameter was represented by $d_{4,3}$. The emulsifiers added were (i) DL (12:0), (ii) DM (14:0), (iii) DP (16:0), (iv) DS (18:0) and (v)DO (18:1), respectively.

Fig. 2-4a (iii) shows the appearance of all the hydrocarbon emulsions added with DP (C16:0). Slight creaming and creaming were observed for C10 emulsion and for C12, C14, C16 and C18 emulsion, respectively. Phase separation was observed for all the emulsions. Among all the emulsifiers (DL-DO), DP appeared to have the strongest destabilizing effects for the protein-stabilized hydrocarbon emulsions because the phase separation was observed in all the emulsions without exception (Fig 2-4a. (i)-(v)). Time dependent changes of mean particle size were observed among all the emulsions (two-way ANOVA, $p < 0.05$). Mean diameter of oil droplets for C14 and C16 emulsion was significantly larger than that for C10, C12 and C18 emulsion 60 min after the preparation of sample emulsions (Tukey's method, $p < 0.05$) (Fig. 2-4b (iii)).

Fig. 2-4a (iv) shows the appearance of hydrocarbon oil emulsions added with DS (C18:0). For creaming, C14 and C16 emulsion were destabilized by DS and C10, C12 and C18 emulsion were slightly destabilized. Phase separation was observed for C12, C14 and C16 emulsion. For the particle size, periodical increase was observed for all the emulsions (two-way ANOVA, $p < 0.05$) (Fig. 2-4b (iv)). It was shown that mean diameter of oil droplets for C16 and C18 emulsion was significantly larger than that for C10, C12 and C14 emulsion after 60 min storage (Tukey's method, $p < 0.05$).

Fig. 2-4a (v) indicates the appearance of hydrocarbon oil emulsions added with DO (C18:1). Slight creaming and creaming occurred in C12 and C14 emulsion, and C16 emulsion, respectively. Phase separation was observed only for C16 emulsion. Periodical changes of the oil droplet diameter were observed for all the emulsions (two-way ANOVA, $p < 0.05$) (Fig. 2-4b (v)). The particle size of C16 emulsion was the largest of five emulsions after 60 min storage (Tukey's method, $p < 0.05$). These data obtained at 60 ºC was similar to those obtained at 35 ºC (Fig. 2-3b), although the increases of oil droplet diameter were larger at higher temperature.

Since the various hydrocarbons with different densities were used as dispersed phases, the

difference of density might potentially affect the creaming velocity. Density of the hydrocarbons (C10-C18) in this research was 0.6996, 0.7198, 0.7346, 0.7459 and 0.7548 g/ml at 60 ℃, respectively (Small, 1986c). The maximum difference was about 0.05. According to the Stoke's law, creaming velocity is in proportion to the second power of particle size, while it is in proportion to the first power of density (Dickinson, 1992a). Therefore, the density difference far less affects the creaming velocity than the difference of particle size. In this work, the difference of mean particle size after destabilization was far larger than 0.05. Based on the fact, the author was able to ignore the difference of densities.

Again, the data obtained in this section was summarized in Table 2-2. DL destabilized C10 and C16 emulsion via creaming process without phase separation and coalescence of oil droplets. DM particularly destabilized C10, C12 and C14 emulsion, which was confirmed by all the emulsion stability tests (creaming, phase separation and microscopically-observed coalescence). DP intensively destabilized C14 and C16 emulsion. DS and DO effectively destabilized C16 emulsion.

The results of this paper suggest that molecular structural similarity between dispersed oil molecules and emulsifiers, i.e., the similarity of carbon chain length between hydrocarbon oil molecules and fatty acid of emulsifiers, may affect the emulsion stability. Sakamoto, Ohba, Kuriyama, Maruo, Ueno & Sato (2004) reported that the addition of emulsifiers with long-chain fatty acid molecules more effectively promotes the crystallization of oil droplets of palm mid fraction (palmitic acid and oleic acid rich), possibly due to the template effects of the added emulsifiers. It is possible that emulsifiers strongly interact with oil molecules in the interfacial region through Van der Waals force when they are similar to each other.

Table 2-2. Summary of the result from combination tests of five hydrocarbons and five DF on the emulsion destabilization

	DL			DM			DP			DS			DO		
	C^{*1}	P^{*2}	M^{*3}	C	P	M	C	P	M	C	P	M	C	P	M
C10	●			●	●	●	▲	●		▲					
C12				●	●	●	●	●		▲	●		▲		
C14				●	●	●	●	●	●	●	●		▲		
C16	●			▲			●	●	●	●	●	●	●	●	●
C18							●	●		▲		●			

*1: Creaming stability (C) was reported as no creaming (no mark), slight creaming (▲) or creaming (●).

*2: Phase separation (P) was expressed as no separation (no mark) or separated (●).

*3: Microscopically-observed coalescence (M) was evaluated by a laser-diffraction particle size analyzer, followed by two-way ANOVA with/without Tukey's comparison method to examine the difference between significantly destabilized emulsions (●) and the others (no mark).

REFERENCES

Álvarez, J. M. & Rodríguez Patino, J. M. (2006). Formulation engineering of food model foams containing diglycerol esters and β-Lactoglobulin. *Industrial & Engineering Chemistry Research* 45, 7510-7519.

Álvarez, J.M., Ruíz-Henestrosa, V. P., Sánchez, C.C. & Rodríguez Patino, J.M. (2008). Role of static and dynamic characteristics of diglycerol esters and β-lactoglobulin mixed films on foaming. 2: adsorption and foaming. *Food Hydrocolloids* 22, 1298-1309.

Bremer, L.G.B., Bijsterbosch, B.H., Walstra, P. & van Vliet, T. (1993). Formation, properties and fractal structure of particle gels. *Advances in Colloid and Interface Science* 46, 117-128.

Chen, J. & Dickinson, E. (1999). Effect of monoglycerides and diglycerol-esters on viscoelasticity of heat-set whey protein emulsion gels. *International Journal of Food Science and Technology* 34, 493–501.

Dickinson, E. (1992a). *An introduction to food colloids*, Oxford University Press, Oxford, UK. Chapter 4, pp.79-122.

Dickinson, E. (1992b). *An introduction to food colloids*, Oxford University Press, Oxford, UK. Chapter 6, pp.140-173.

Dickinson, E. & McClements, D.J. (1996). *Advances in Food Colloids*, Blackie Academic and Professional, London. Chapter 7, pp.211-246.

Engelkirk, P.G. & Engelkirk, J.D. (2007). *Laboratory Diagnosis of Infectious Diseases: Essentials of Diagnostic Microbiology*. Lippincott Williams & Wilkins, Pennsylvania. chapter 10, pp. 240-271.

Holstborg, J., Pedersen, B.V., Krog, N. & Olesen, S.K. (1999). Physical properties of diglycerol esters in relation to rheology and stability of protein-stabilised emulsions. *Colloids and*

Surfaces B: Biointerfaces 12, 383-390.

Kabara, J.J., Swieczkowski, D.M., Conley, A.J. & Truant, J.P. (1972). Fatty acids and derivatives as antimicrobial agents. *Antimicrobial Agents Chemotherapy* 2, 23-28.

Kato, N. & Shibasaki, I. (1975). Comparison of antimicrobial activities of fatty acids and their esters. *Journal of Fermentation Technology* 53, 793-801.

Krog, N. (1984). Thermodynamics of interfacial films in food emulsions. In: *Microemulsions and Emulsions in Foods*, ACS symposium series 448, chapter 11, pp.138-145.

Kumar, T. N., Sastry, Y. S. R. & Lakshminarayana, G. (1989). Preparation and surfactant properties of diglycerol esters of fatty acids. *Journal of the American Oil Chemists' Society* 66, 153-157.

Lowry, O. H., Rowebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *The Journal of Biological Chemistry* 193, 265-275.

Matsumiya, K., Takahashi, Y., Nakanishi, K., Dotsu, N. & Matsumura, Y. (2007). Destabilization of O/W emulsions by the diglycerol ester. In: *Proceedings of the 98th AOCS Annual Meeting and Expo, Quebec, Canada*.

Matsumiya, K., Takahashi, W., Inoue, T. & Matsumura, Y. (2010). Effects of bacteriostatic emulsifiers on stability of milk-based emulsions. *Journal of Food Engineering* 96, 185–191.

McClements, D.J. (2004). Protein-stabilized emulsions. *Current Opinion in Colloid and Interface Science* 9, 305–313.

McClements, D.J. (2005). *Food Emulsions: Principles, Practice, and Techniques, second ed*. CRC Press, Boca Raton, Florida. Chapter 7, pp. 269-339.

Morrison, R.T. & Boyd, R. N. (1992). *Organic Chemistry*, 6th edition, Prentice Hall Inc., New Jersey, USA. Chapter 8, Alkenes I. Structure and preparation, Elimination, pp.273-316.

O'Brien, R. D. (2003). *Fats and Oils: Formulating and Processing for Applications*, second edition, CRC press, Boca Raton, Florida. Chapter 1, Raw Materials, pp.1-55.

Robins, M. M. (2000). Emulsions - creaming phenomena. *Current Opinion in Colloid & Interface Science* 5, 265-272.

Parker, R. (2003). *Introduction to Food Science*. Delmar, NY, USA. chapter 9, pp.141-155.

Rousseau, D. (2000). Fat crystals and emulsion stability. *Food Research International* 33, 3-14.

Sakamoto, M., Ohba, A., Kuriyama, J., Maruo, K., Ueno, S. and Sato, K. (2004). Influences of fatty acid moiety and esterification of polyglycerol fatty acid esters on the crystallization of palm mid fraction in oil-in-water emulsion. *Colloids and Surfaces B: Biointerfaces* 37, 27–33.

Small, D. M. (1986a). *The Physical Chemistry of Lipids: From Alkanes to Phospholipids (Handbook of Lipid Research)*, vol 4, Plenum Press, New York and London. Chapter 1, pp. 1-20.

Small, D. M. (1986b). *The Physical Chemistry of Lipids: From Alkanes to Phospholipids (Handbook of Lipid Research)*, vol 4, Plenum Press, New York and London. Chapter 2, pp. 21-41.

Small, D. M. (1986c). *The Physical Chemistry of Lipids: From Alkanes to Phospholipids (Handbook of Lipid Research)*, vol 4, Plenum Press, New York and London. Appendix I, pp. 559-576.

Yamazaki, K, Yamamoto, T., Kawai, Y. & Inoue, N. (2004). Enhancement of antilisterial activity of essential oil constituents by nisin and diglycerol fatty acid ester. *Food Microbiology* 21, 283–289.

Chapter 3:

Diglycerol esters of fatty acids promote severe coalescence between protein-stabilized oil droplets by emulsifier-protein competitive interactions

1. INTRODUCTION

Food products are subjected to various degradations during long-term shelf-life; *i.e.*, undesirable changes of appearance, off-flavors, or bacterial poisonings. For anaerobic canned-state beverages containing milk ingredients, manufactures particularly have to pay attention to heat–resistant sporeformers including *Bacillus* spp. and *Clostridium* spp (Parker, 2003; Engelkirk & Engelkirk, 2007). The sporeformers are often prevented from growing in the anaerobic condition by unique emulsifiers with bacteriostatic effects, diglycerol esters of fatty acids (Matsumiya, Takahashi, Inoue & Matsumura, 2010). They are categorized as polyglycerol esters of fatty acids, in which the polymerization degree of glycerol and the kind of fatty acids can be flexibly changed. Diglycerol esters of fatty acids are surface active molecules that reduce interfacial tension at the oil-water interface (Holstborg, Pedersen, Krog & Olesen, 1999). On the other hand, in spite of the name "emulsifier", they tend to promote severe coalescence between oil droplets stabilized by milk proteins under agitating conditions (Holstborg *et al.*, 1999).

Coalescence is the process where two or more oil droplets merge together to form a larger oil droplet. It mainly depends on processing of emulsions, environmental conditions around oil droplets, and interfacial structures between oil and water (McClements, 2005a). Mechanical stresses, oil concentration and freeze-thaw treatments promote oil droplet coalescence according to increased collision or contact between oil droplets. Protein emulsifiers adsorbed to the surface of oil droplets effectively retard droplet coalescence by forming protective and repulsive membranes around the oil droplets, while small-molecule emulsifiers prevent the close approach and coalescence of oil

droplets by the Gibbs-Marangoni effects (Dickinson, 1992; Rousseau, 2000; McClements, 2004; Matsumura & Matsumiya, 2012). The composition and structure of the interfacial region, however, do not only depend on the type and concentration of surface-active molecules present during preparation but also depend on the events that occur during and after emulsion preparation, i.e., multilayer formation, covalent bonding or protein displacement at the oil-water interface (McClements, 2005b).

Previous researches revealed that bacteriostatic emulsifiers including diglycerol esters of fatty acids can interact with milk proteins and displaces them from the oil–water interface (Holstborg *et al*., 1999; Chen & Dickinson,1999), probably leading to emulsion instability such as creaming, aggregation or coalescence (Matsumiya *et al*., 2010). For air-water interfaces, foaming stability, e.g, drainage or coalescence of diglycerol esters-milk proteins mixed solutions depends on the emulsifier/protein ratio in the mixture (Álvarez & Rodríguez Patino, 2006; Álvarez, Ruíz-Henestrosa, Sánchez & Rodríguez Patino, 2008). These researches and other recent works indicated that diglycerol esters of fatty acids with various carbon chain lengths have different effects on bacteriostatic activity and stability of air bubbles or emulsions against coalescence (Yamazaki, Yamamoto, Kawai & Inoue 2004; Matsumiya, Nakanishi, Matsumura, 2011). Meanwhile, in our previous study performed on hydrocarbons, it was shown that protein displacement by diglycerol esters of fatty acids tend to cause the coalescence between oil droplets depending on hydrocarbon types (Matsumiya *et al*., 2011).   The underlying mechanisms of oil droplet coalescence caused by the bacteriostatic emulsifier, DO particularly observed for food-oil emulsions are still unclear.

At present, though the emulsifiers cause the destabilization of milk-based emulsions, manufacturers are still required to use them for avoiding the bacterial poisoning of the food products. In this work, we investigate physicochemical and colloidal properties of diglycerol esters

of mono-oleic acid (DO), a representative of the bacteriostatic emulsifiers, that is, interactions between the bacteriostatic emulsifiers and milk proteins in plane interface systems and emulsion systems in order to understand the destabilizing mechanisms of the bacteriostatic emulsifiers. As a systematic approach, physicochemical and colloidal properties of DO are compared to a widely utilized emulsifier in the food industry, monoglycerol esters of mono-oleic acid (MO) and triglycerol esters of mono-oleic acid (TO), both of which do not cause the destabilization of protein-based emulsions.

2. MATERIALS AND METHODS

2.1 Materials

Corn oil was obtained from Nacalai tesque, Inc. (Kyoto, Japan). Soybean oil was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Corn oil was used for tensiometry and determination of free oil in Section 2.3.1 and 2.3.2.1. Soybean oil was used for all the other experiments. MO (monoester content > 93%), DO (monoester content > 87%), TO (monoester content > 85%) and sodium caseinate (Na-CN) were provided by Riken Vitamin Co., Ltd. (Tokyo, Japan). Hydrophile-lypophile balance (HLB) of MO, DO and TO are 4.3, 7.4 and 9.4, respectively. Deionized water was used for the preparation of all the solutions and emulsions.

2.2 Plane interface system

2.2.1 Tensiometry

Na-CN was dissolved into aqueous phase at 60°C. MO and DO were dissolved into oil phase at 60 °C, while DO and TO were dispersed into aqueous phase using a high-speed blender (Physcotron, NS-51, Microtec Co.,Ltd, Japan) at 19,300 rpm for 2 minutes at 60 °C as DO can be dispersed into both oil phase and aqueous phase. The pH of the aqueous solutions was approximately from 6 to 7. These emulsifiers were added into oil phase (50 wt%) or water phase (50 wt%) to give additional 0.0 - 0.1 wt% of emulsifier to the added phase; for example, 0.1 wt% of

MO (25 mg) was dissolved into corn oil (25 g) followed by pouring onto water (25 g) and subjected to subsequent measurements. The interfacial tension between corn oil and water either of which includes MO, DO, TO or Na-CN was measured using the Wilhelmy Plate method (Automatic Surface Tensiometer, CBVP-A3, Kyowa Interface Science Co., Ltd, Japan) at 20 °C.

2.2.2 Phase-to-phase migration

The emulsifiers were dissolved into oil phase or dispersed into aqueous phase according to the method shown in 2.2.1. Final concentrations of the emulsifiers were 0.2 wt% to the total weight of oil and water phases; e.g., 0.2 wt% of DO (100 mg) was dissolved into oil phase (25 g) followed by pouring onto water (25 g). The oils were gently poured onto the aqueous solutions. These samples were stored in an incubator at 60 °C for 24 h. A fraction of aqueous phase was carefully transferred into a tared vessel using a syringe followed by a freeze-drying process. The remained emulsifier was weighed and then migration degree was calculated based on the weight. Emulsifier concentration in the oil-based system was evaluated by difference of the added emulsifier weight and the emulsifier weight present in the aqueous phase.

2.3 Emulsion system

2.3.1 Emulsifying ability of the emulsifiers without proteins

The emulsifiers were dissolved into oil phase or dispersed into aqueous phase according to the method shown in 2.2.1. Final concentrations of the emulsifiers were 0.2 wt% to the total weight of oil and aqueous phases. The mixture of oil (50 wt%), water (50 wt%) and emulsifier (+0.2 wt%) was homogenized using the high-speed blender at 19,300 rpm for 3 minutes at 60 °C. The amount of free non-emulsified oil was determined according the method described before with slight modifications (Palanuwech, Potineni, Roberts & Coupland, 2003). Corn oil containing red dye (0.0010 wt% of Oil Red O) was poured onto emulsions and then gently mixed. A fraction of the dyed oil was taken into a sample tube followed by a centrifugation at 87,000g for 15 minutes at

20 °C (ultracentrifuge CS120, Hitachi Koki Co., Ltd., Tokyo, Japan). The absorbance of the transparent oil layer was measured at 517 nm using a UV visible spectrophotometer (UV-2400 PC, Shimadzu Corp., Japan). The percentage of free oil (Φ_d) is given by the equation:

$\Phi_d = m_0 (a-1)/ m_e \Phi$

where m_0 is the mass of added dye solution, m_e is the mass of emulsion, Φ is the mass fraction of oil in the emulsion and a is the ratio of the measured absorbance of the dye solution (by definition, $a \geq 1$).

2.3.2 Relationship between emulsion destabilization and emulsifier-protein competitive interaction

2.3.2.1 Sample preparation

Demulsification caused by the water-soluble small-molecule emulsifiers was tested in a two-step processing as follows. Na-CN was added into 40 wt% of water to give additional 0.2 wt% to the total weight of oil and water, while DO and TO were dispersed into 10 wt% of water to give additional 0.0 - 0.4 wt% to the total weight of oil and water, respectively. The protein solution and 50 wt% of soybean oil were homogenized using the high-speed blender at 19,300 rpm for 3 minutes at 60 °C, which was subsequently mixed with the DO and TO emulsifier dispersions followed by a high-shear destabilizing process using the high-speed blender at 19,300 rpm for 3 minutes at 60 °C.

Destabilizing effects of the oil-soluble MO and DO emulsifiers were evaluated in a single-step processing as follows because it is difficult to make them distribute into dispersed oil phase of oil-in-water emulsions after emulsion formation. Na-CN was dissolved into 50 wt% of water to give additional 0.2 wt% to the total weight of oil and water, while MO and DO were dispersed into 50 wt% of soybean oil to give additional 0.0 - 0.4 wt% to the total weight of oil and water, respectively. The protein solution and soybean oil including the oil-soluble emulsifiers were mixed and then homogenized using the high-speed blender at 19,300 rpm for 3 minutes at 60 °C.

Just after the emulsion preparation, phase separation resulting from severe coalescence of oil droplets was observed for emulsions including the bacteriostatic emulsifiers, DO. All the prepared emulsions were placed in an incubation room at 20 °C overnight to ensure almost complete separation between oil and aqueous phases. Clearly separated oil phase or opaque cream layer at the top and turbid serum layer at the bottom were confirmed after the storage. A small fraction of the serum layer was transferred to a sample tube using a syringe, and then diluted 4-times with deionized water. This diluted solution was centrifuged at 87,000g for 20 min at 20 °C using an ultracentrifuge (CS120, Hitachi Koki Co., Ltd., Japan). It was separated into opaque cream layer at the top and transparent layer at the bottom. The transparent layer was used for evaluation of adsorbed proteins in the next section. The destabilized emulsion left was subjected to evaluation of destabilized oil. The amount of destabilized free oil expressed as wt% was determined according to the method described in Section 2.3.1.

2.3.2.2 Evaluation of proteins adsorbed to the oil droplet surface

The amount of proteins adsorbed onto the surface of oil droplets were evaluated measuring protein concentrations in the obtained transparent layer by Lowry's method using Na-CN as a standard (Lowry, Rowebrough, Farr & Randall, 1951). The relative amount of proteins adsorbed to the oil droplet surface was calculated from the protein content of the layer.

2.4 Statistical analysis

All experiments were conducted in triplicate or more with freshly prepared samples. Statistical analyses were performed using Microsoft Excel ver. 2010 for Windows.

3. RESULTS AND DISCUSSION

3.1 Plane interface system

3.1.1 Tensiometry

Surface active molecules such as small-molecule emulsifiers and proteins adsorb to the

oil-water interface to reduce the interfacial tension. The reduced interfacial tension is responsible for formation of fine emulsions or a decreased stability of emulsion droplets to coalescence (Wilde, 2000). We measured the interfacial tension between corn oil and deionized water as a function of emulsifier concentration in the specified phase to estimate adsorption behavior of the surface active compounds used in this research.

Figure 3-1 shows concentration-dependent interfacial tension between oil and water phases using MO, DO, TO or Na-CN as an emulsifier measured by the Wilhelmy plate method at 20 °C. The interfacial tension between corn oil and water without any added surface-active molecules was 24.1 mN/m, which was a reasonable value corresponding to a literature (Gaonkar, 1989). Interfacial tension between oil and water phases varied with type and concentration (0.01 - 0.10 wt%) of the emulsifiers (two-way ANOVA, $p<0.05$), although decreased according to the increase of all the emulsifiers including the milk proteins, clearly indicating that all the emulsifiers including the milk proteins adsorbed to the oil-water interface. The polyglycerol ester of oleic acid used in this work more effectively reduced the interfacial tension with an increase of HLB; that is, surface activity was in order of polymerization degree of glycerols, TO > DO > MO. Such a tendency is agreeable to the data measured for MO and DO dissolved into oil phase reported by Holstberg *et al* (1999).

In general, emulsions prepared by emulsifiers with an intermediate HLB value (HLB = 6 - 8) are unstable to coalescence because the interfacial tension between oil and water becomes so low that little energy is required to disrupt the interfacial membrane (McClements, 2008). DO with a HLB value of 7.4 particularly dissolved in oil lowered the interfacial tension close to zero at 0.10 wt%, whilest TO more efficiently lowered the interfacial tension than DO at all the concentrations (Figure 3-1). Considering that TO is empirically known not cause the severe coalescence of oil droplets in the emulsion, the emulsifier-induced oil droplet coalescence by DO cannot be necessarily explained by the tensiometric theory. Some very surface active emulsifiers with low
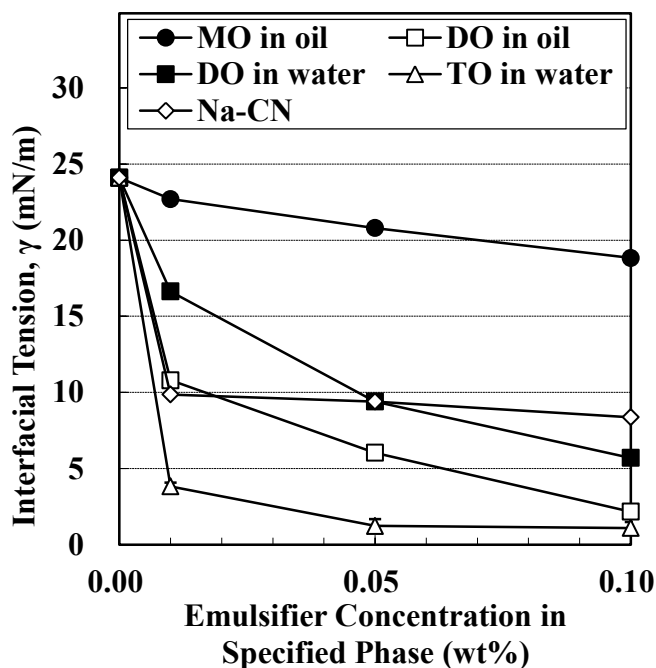
Figure 3-1. Interfacial tension between corn oil and water containing various concentrations of surface active compounds measured by the Wilhelmy plate method at 20 °C (mean ± SD, n = 3).

interfacial tensions like TO are very resistant to coalescence, particularly those with high surface charge or steric properties.

It should be noted that DO dissolved in the oil phase more markedly lowered the interfacial tension than DO dispersed in the aqueous phase at each concentration. Since the concentration-dependent interfacial tension was measured under equilibrium states after enough adsorption time, the different adsorption behavior of DO to the oil-water interface can be attributed not to diffusion rate from the originally added phase to the interface, but to molecular preference of DO to the originally added phases or to molecular interactions of DO at the oil-water interface. DO with polar hydroxyl groups, ether bonding and ester bonding dissolved into oil phase may tend to preferably move to the oil-water interface rather than to stay in the original phase compared to DO dispersed in aqueous phase. Otherwise, there is a possibility that the polar groups and bondings of DO previously adsorbed to the oil-water interface form a hydrated layer around them to disturb efficient approaches of DO in the aqueous phase through steric repulsion.
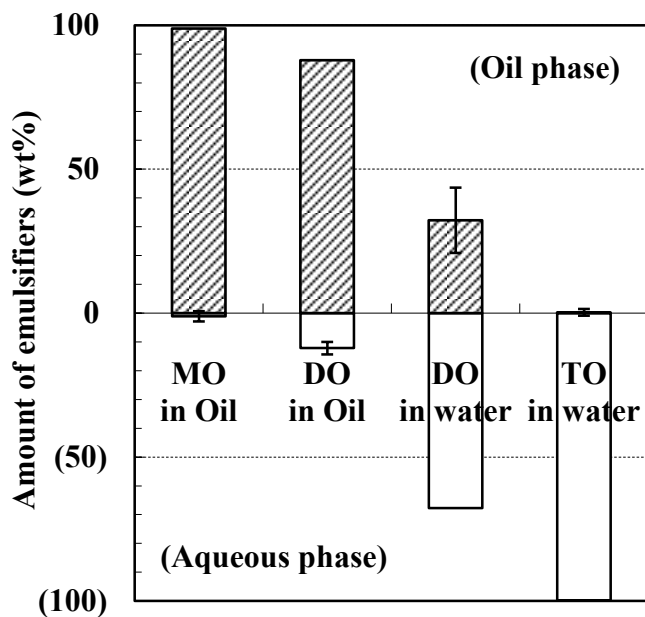
Figure 3-2. Degree of phase-to-phase migration of MO, DO and TO between oil phase (soybean oil) and aqueous phase at 60 °C for 24 h as determined by freeze dry (mean ± SD, n = 3).

MO and TO were dissolved and dispersed in oil and aqueous phase, respectively. DO was dissolved and dispersed in both oil and aqueous phase, respectively. Emulsifiers present in oil and aqueous phase were plotted above and below the zero line, respectively.

3.1.2 Measurement of phase-to-phase migration

In order to evaluate molecular preference of DO and the other emulsifiers to oil and aqueous phases, we measured phase-to-phase migration of the emulsifiers, dissolving and dispersing each of them into oil and aqueous phase, respectively. Since DO can be readily dispersed in both oil and aqueous phases, different from MO and TO that can be readily dissolved only in oil and aqueous phase, respectively, DO was added into both the phases. Despite of the tensiometry performed at 20 °C, we carried out these measurements at 60 °C to promote the phase-to-phase migration of the emulsifiers and to relate the obtained results to the estimation of emulsifier migration under destabilizing conditions by the high-speed blender to be discussed in the section of emulsion system.

Figure 3-2 indicates degree of phase-to-phase migration of the emulsifiers used in this research from oil phase to aqueous phase and vice versa at 60 °C for 24 h. The degree of phase-to-phase migration of the emulsifiers were different from each other (one-way ANOVA, $p <$

0.05). After 24 h, MO dissolved in the oil phase and TO dispersed in the aqueous phase scarcely moved and then stayed in the originally added phases, clearly indicating that MO and TO tend to prefer oil or water as expected by their HLB values, that is, 4.3 for MO and 9.4 for TO (Dickinson & McClements, 1995).

DO added into both oil phase and aqueous phase migrated to the other phase in contrast to MO and TO (Figure 2). DO dispersed in water migrated to the other phase to larger extent than DO in oil at 60°C for 24 hours. As an additional experiment, we also measured the phase-to-phase migration of DO added in both the phases at 20°C for 4 weeks. Resultantly, 14.6 ± 1.5 wt% of DO dissolved in oil phase and 19.1 ± 2.9 wt% of DO dispersed in water phase migrated to the other phase, corresponding to the tendency from the data obtained at 60°C; that is, the migration degree of DO in water was more than that in oil. These data suggest that different results obtained from DO dissolved in oil and water can be attributed to an energy barrier against the formation of self-organization structure such as liposome or micelle that can be visually observed as opaque layer in an aqueous phase. The obtained data obviously indicate that DO prefers to stay in oil phase rather than water phase and agree with the discussion in the tensiometry section. On the other hand, they do not necessarily agree with a well-known fact that surfactant with an intermediate HLB value about 7.0 has no preference for either oil or water (Sangwal, 2007; McClements, 2008).

3.2 Emulsion system

The author characterized the emulsifiers from the viewpoint of their emulsifying ability and competitive interactions between the milk proteins to clarify their roles in the emulsion system in this section.

3.2.1 Emulsifying ability of the emulsifiers without proteins

As a first step, non-emulsified free oil in the emulsions including 50 wt% of soybean oil and 50 wt% of water after the emulsification processes was measured to evaluate emulsifying ability of
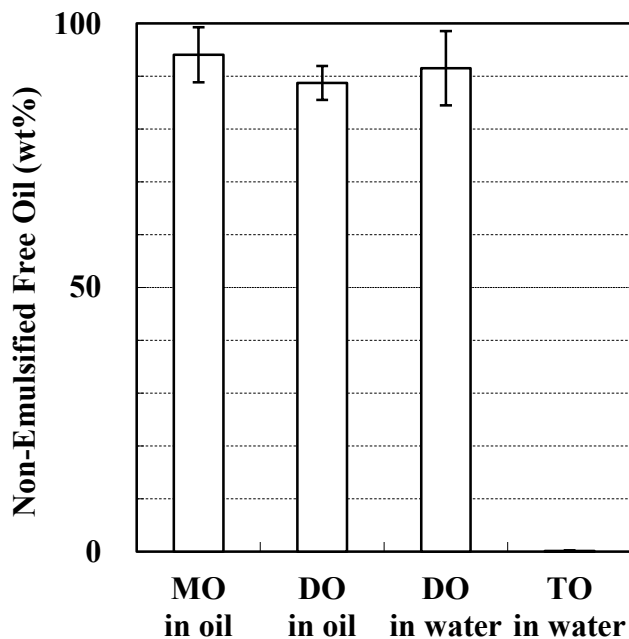
Figure 3-3. Non-emulsified free oil of emulsions to the original amount of oil containing MO, DO and TO added in oil or water without Na-CN after emulsification processes at 60 °C (mean ± SD, n = 3).

Soybean oil and water was homogenized at the weight ratio of 1:1 with additional 0.2 wt% of each emulsifier to the total weight of the oil and water.

MO, DO and TO without the proteins (Figure 3-3). The amount of non-emulsified free oil was significantly different according to the type of emulsifiers (one-way ANOVA, $p < 0.05$). Non-emulsified free oil in the emulsions prepared by TO was close to zero; that is, 50 wt% of soybean oil can be completely emulsified in the equivalent weight of water by TO. For MO and DO, most of the oil was in free bulk states in the emulsions prepared by the emulsifiers regardless of the dispersed phase. These results indicate that TO has enough ability to emulsify soybean oil by itself and that MO and DO lack an ability to form oil-in-water emulsions by themselves.

Based on the HLB concept (McClements, 2005c; McClements, 2008), emulsifiers with a high HLB value (8 - 18) and a low HLB value (4 - 6) stabilize O/W type emulsions and W/O type emulsions, respectively and thereby the evaluated emulsifying ability of TO and MO is considered to be reasonable. DO is well-known to favorably form liposomes (Holstborg et al., 1999; Pitzalis, Monduzzi, Krog, Larsson, Ljusberg-Wahren & Nylander, 2000; Johnsson, Lam, Barauskas & Tiberg, 2005) which implies that the critical packing parameter value is nearly 1.0 (Yaghmur, de Campo, Sagalowicz, Leser & Glatter, 2006). Emulsifiers with such characteristics favor not only the formation of planar membrane as a self-organization structure but also the formation of planar
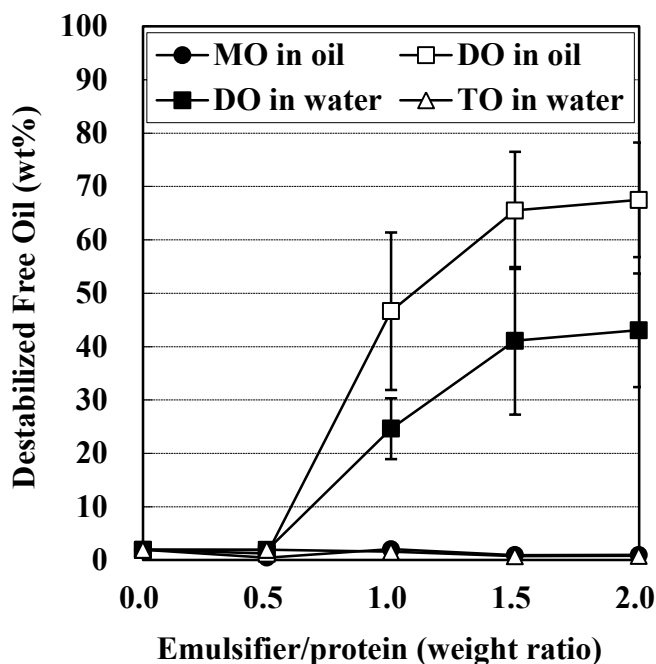
Figure 3-4. Destabilized free oil to the original amount of oil in protein-based emulsions as a function of different emulsifier/protein weight ratio (mean ± S.D. n = 6).

The amount of the protein, sodium caseinate was fixed at 0.2 wt% to the total weight of oil and water.

oil-water interface when they adsorb to the interface in contrast to other types of emulsifiers (Israelachvili, 2011). In this context, the author speculated that the bacteriostatic emulsifier, DO may predominantly occupy the oil droplet surfaces with large curvature in the emulsion to make them in more planar states, i.e., severe coalescence of oil droplets leading to phase separation of oil and water. The author evaluated destabilization of the protein-based emulsions and emulsifier-protein competitive interactions on the oil droplet surfaces regarding the surface curvature in the next experiments.

3.2.2   Evaluation of emulsion destabilization

The amount of destabilized free oil in 50 wt% soybean oil emulsions as a function of different emulsifier/protein ratio was measured to evaluate emulsion destabilization caused by the small molecule emulsifiers (Figure 3-4). The amount markedly varied with type and weight ratio (0.5 - 2.0) of the emulsifiers (two-way ANOVA, $p < 0.05$). Emulsions prepared only by Na-CN were so stable that free oil was not detected (emulsifier/protein = 0.0). Little amount of destabilized oil was observed for emulsions including MO and TO at all the emulsifier/protein weight ratio,
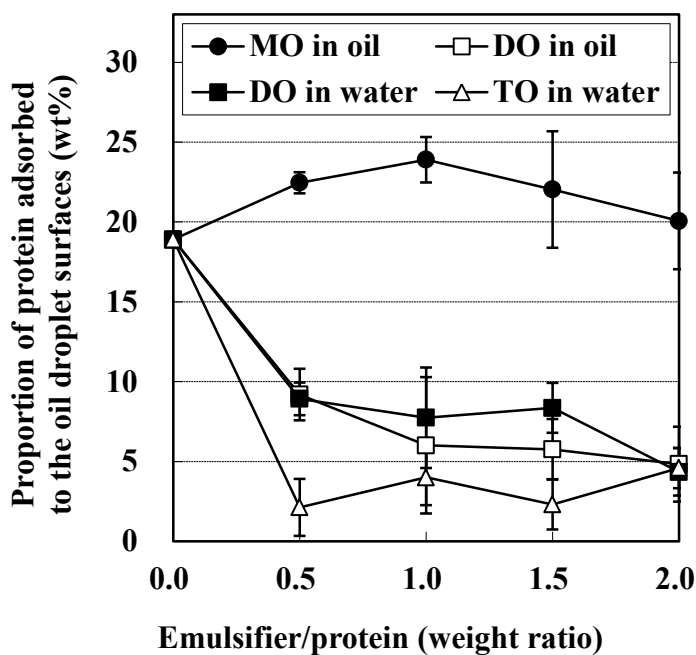
Figure 3-5. Proportion of protein adsorbed to the oil droplet surfaces as a function of different emulsifier/protein weight ratio (mean ± S.D. n = 6).

The amount of the protein, Na-CN was fixed at 0.2 wt% to the total weight of oil and water.

indicating that both the emulsifiers do not cause the emulsion destabilization. Conversely, the amount of destabilized oil was increased according to the increase of DO dispersed in both oil and water. More amount of destabilized free oil was measured in the emulsions prepared with DO dissolved in oil than those with DO dispersed in water. These results can be related to the data obtained by the tensiometry in Figure 3-1 that DO dissolved in oil more efficiently lowered the interfacial tension between oil and water than DO dispersed in water. They can also be related to the different degrees of phase-to-phase migration between DO in oil and water shown in Figure 3-2. DO added in oil with a tendency to stay at the oil-water interface may cause the severe coalescence between oil droplets.

3.2.3 Evaluation of proteins adsorbed to the oil droplet surfaces

The amount of proteins adsorbed to the oil droplet surfaces as a function of different emulsifier/protein ratio was measured to evaluate competitive interactions between the milk proteins and the small molecule emulsifiers, that is, competitive adsorption to the oil-water interface and protein displacement from the oil droplet surfaces (Figure 3-5). The amount of adsorbed

proteins significantly changed according to the type and weight ratio (0.5 - 2.0) of the emulsifiers (two-way ANOVA, $p < 0.05$). When the emulsions were prepared by only the milk proteins, 18.9 wt% of added proteins adsorbed to the oil-water interface. Addition of MO into the oil phase increased the adsorbed proteins up to 23.9 wt% within the emulsifier/protein ratio from 0.5 to 2.0. It is known that widely-used oil soluble emulsifiers are less effective at preventing the protein adsorption (Dickinson & Tanai, 1992; Euston, Singh, Munro & Dalgleish, 1995) and, moreover, that they sometimes raise the adsorption of proteins to the oil-water interface (Dickinson, Owusu, Tan & Williams, 1993). Dickinson *et al*. (1993) also reported the characteristic effect of a similar emulsifier, monoglycerol ester of mono-stearic acid (glycerol monostearate) to increase the amount of whey proteins on the oil droplet surfaces possibly due to fast configurational arrangements of adsorbed protein by the emulsifier or some direct attractive interactions between the emulsifier and the proteins. The increased proteins by the addition of MO seemed to contribute to the stability of protein-based emulsions against oil droplet coalescence in this research (Figure 3-4).

TO with a high HLB value almost completely displaced the adsorbed proteins from the oil droplet surfaces at all the emulsifier/protein weight ratios (Figure 3-5). Water soluble emulsifiers are capable of efficiently remove adsorbed protein from the oil-water interface even when they are added after emulsification processes (Dalgleish, 2004). Since emulsion destabilization such as severe coalescence or phase separation was not observed for emulsions including Na-CN and TO (Figure 3-4), TO with high emulsifying ability (Figure 3-3) was likely to contribute to the stabilization of the emulsions rather than Na-CN.

DO added both into oil and water reduced the adsorbed proteins in a similar manner to TO, while MO was less effective at displacing the proteins under the tested conditions (Figure 3-5). These results suggest that emulsifiers with an intermediate HLB value that can be dissolved and dispersed both in oil and water tend to behave like those with not a low HLB value but a high HLB
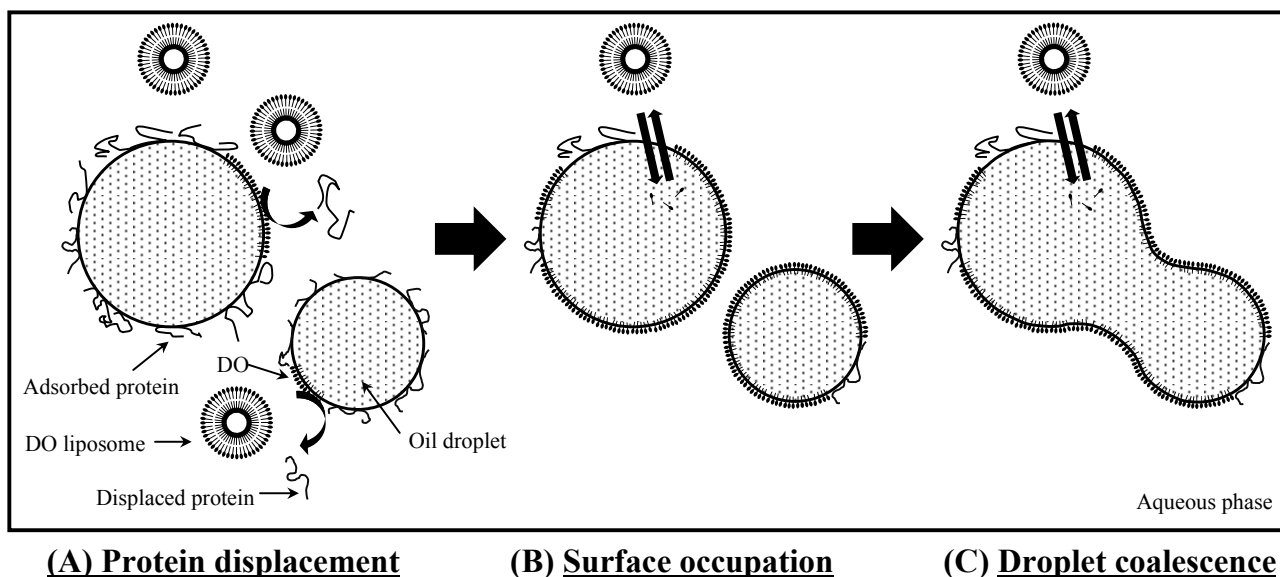
(A) Protein displacement   **(B) Surface occupation**   **(C) Droplet coalescence**

Figure 3-6. A schematic diagram of the destabilizing mechanism of DO dispersed in water.

Via the emulsifier-protein competitive interactions (A), DO predominantly occupies the oil droplet surfaces (B) to promote severe coalescence of emulsion oil droplets through the active migration of DO and the formation of planar interface (C).

value with regard to the protein displacement on the oil droplet surfaces. DO was slightly more effective at displacing the proteins when it was dissolved in oil than dispersed in water, corresponding to the results from the tensiometric measurements (Figure 3-1). Comparing the emulsion destabilization by DO (Figure 3-4) and the DO-protein competitive interactions from the emulsifier/protein ratio of 0.5 to 2.0 (Figure 3-5), destabilized free oil was increased according to a decrease of adsorbed protein (r = -0.82). The reason why little destabilized oil was observed at emulsifier/protein ratio of 0.5 may be that this concentration of DO was enough to interact with the proteins but insufficient for covering the oil droplet surfaces to destabilize the emulsions.

4. CONCLUSION

Our data show that DO with little emulsifying ability (Figure 3-3) predominantly occupies the oil droplet surfaces via the emulsifier-protein competitive interactions to promote severe coalescence of emulsion oil droplets probably because DO actively migrates between oil and

aqueous phase (Figure 3-2) and it favors to form the planar oil-water interface when they adsorb to the interface. A schematic diagram of the destabilizing mechanism of DO dispersed in water is shown in Figure 3-6.

REFERENCES

Álvarez, J. M. & Rodríguez Patino, J. M. (2006). Formulation engineering of food model foams containing diglycerol esters and β-lactoglobulin. *Industrial & Engineering Chemistry Research* 45, 7510-7519.

Álvarez, J. M., Ruíz-Henestrosa, V. P., Sánchez, C. C. & Rodríguez Patino, J. M. (2008). Role of static and dynamic characteristics of diglycerol esters and β-lactoglobulin mixed films on foaming. 2: adsorption and foaming. *Food Hydrocolloids* 22, 1298-1309.

Chen, J. & Dickinson, E. (1999). Effect of monoglycerides and diglycerol-esters on viscoelasticity of heat-set whey protein emulsion gels. *International Journal of Food Science and Technology* 34, 493-501.

Dalgleish, D. G. (2004). *Food emulsions*, fourth edition, revised and expanded, Friberg, S. E.; Larsson, K.; Sjöblom, J., Eds., Marcel Dekker, New York, USA. pp.1-44.

Dickinson, E. & McClements, D. J. (1995). *Advances in Food Colloids*, Chapman & Hall, London. pp. 247-279.

Dickinson, E. (1992). *An introduction to food colloids*. Oxford University Press, Oxford, UK. pp. 140-173.

Dickinson, E., Owusu, R. K., Tan, S. & Williams, A. (1993). Oil-soluble surfactants have little effect on competitive adsorption of α-lactalbumin and β-lactoglobulin in emulsions. *Journal of Food Science* 58, 295-298.

Dickinson, E. & Tanai, S. (1992). Protein displacement from the emulsion droplet surface by oil-soluble and water-soluble surfactants. *Journal of Agricultural and Food Chemistry*, 40, 179-183.

Engelkirk, P. G. & Engelkirk, J. D. (2007). *Laboratory diagnosis of infectious diseases: Essentials of diagnostic microbiology*. Lippincott Williams & Wilkins, Pennsylvania. pp. 240-271.

Euston, S. E., Singh, H., Munro, P. A. & Dalgleish, D. G. (1995). Competitive adsorption between sodium caseinate and oil-soluble and water-soluble surfactants in oil-in-water emulsions. *Journal of Food Science* 60, 1124-1131.

Gaonkar, A. G. (1989). Interfacial tensions of vegetable oil/water systems: Effect of oil purification. *Journal of the American Oil Chemists' Society* 66, 1090-1092.

Holstborg, J., Pedersen, B. V., Krog, N. & Olesen, S. K. (1999). Physical properties of diglycerol esters in relation to rheology and stability of protein-stabilised emulsions. *Colloids and Surfaces B: Biointerface* 12, 383-390.

Israelachvili, J. N. (2011). *Intermolecular and Surface Forces*, third edition, Academic Press, USA. pp. 535-576.

Johnsson, M., Lam, Y., Barauskas, J. & Tiberg, F. (2005). Aqueous phase behavior and dispersed nanoparticles of diglycerol monooleate/glycerol dioleate mixtures. *Langmuir* 21, 5159-5165.

Lowry, O. H., Rowebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *The Journal of Biological Chemistry* 193, 265-275.

Matsumiya, K., Takahashi, W., Inoue, T. & Matsumura, Y. (2010). Effects of bacteriostatic emulsifiers on stability of milk-based emulsions. *Journal of Food Engineering* 96, 185-191.

Matsumiya, K., Nakanishi, K. & Matsumura, Y. (2011). Destabilization of protein-based emulsions by diglycerol esters of fatty acids - The importance of chain length similarity between

dispersed oil molecules and fatty acid residues of the emulsifier. *Food Hydrocolloids* 25, 773-780.

Matsumura, Y. & Matsumiya, K. (2012). *Food Proteins and Peptides: Chemistry, Functionality, Interactions, and Commercialization,* Hettiarachchy, N. S., Sato, K., Marshall, M. R., Kannan, A., Eds., CRC Press, Boca Raton, Florida. pp. 125-150.

McClements, D. J. (2004) Protein-stabilized emulsions. *Current Opinion in Colloid and Interface Science* 9, 305-313.

McClements, D. J. (2005a). *Food Emulsions: Principles, practice, and techniques*, second ed.; CRC Press: Boca Raton, Florida. pp. 269-339.

McClements, D. J. (2005b). *Food Emulsions: Principles, practice, and techniques*, second ed.; CRC Press: Boca Raton, Florida. pp. 175-232.

McClements, D. J. (2005c). *Food Emulsions: Principles, practice, and techniques*, second ed.; CRC Press: Boca Raton, Florida. pp. 95-174.

McClements, D. J. (2008). *Food Lipids: Chemistry, Nutrition, and Biotechnology*, third edition, Akoh, C. C., Min, D. B., Eds., CRC Press, Boca Raton, Florida. pp. 63-98.

Palanuwech, J., Potineni, R., Roberts, R. F. & Coupland, J.N. (2003). A method to determine free fat in emulsions. *Food Hydrocolloids* 17, 55-62.

Parker, A. (2003). Heat. *Introduction to food science*, Delmar, New York. pp. 141-155.

Pitzalis, P. Monduzzi, M., Krog, N., Larsson, H., Ljusberg-Wahren, H. & Nylander, T. (2000). Characterization of the liquid-crystalline phases in the glycerol monooleate/diglycerol monooleate/water system. *Langmuir* 16, 6358-6365.

Rousseau, D. (2000). Fat crystals and emulsion stability - a review. *Food Research International* 33, 3-14.

Sangwal, K. (2007). *Additives and Crystallization Processes: From Fundamentals to Applications*, John Wiley & Sons Ltd., West Sussex, England. pp.1-20.

Wilde, P. J. (2000). Interfaces: their role in foam and emulsion behavior. *Current Opinion in Colloid & Interface Science* 5, 176-181.

Yaghmur, A., De Campo, L., Sagalowicz, L., Leser, M. E. & Glatter, O. (2006). Control of the internal structure of MLO-based isasomes by the addition of diglycerol monooleate and soybean phosphatidylcholine. *Langmuir* 22, 9919-9927.

Yamazaki, K., Yamamoto, T., Kawai, Y., & Inoue, N. (2004). Enhancement of antilisterial activity of essential oil constituents by nisin and diglycerol fatty acid ester. *Food Microbiology* 21, 283-289.

Chapter 4

Evaluation of long-term stability of milk beverages by a novel method for rapid determination of aggregation forces between colloidal particles

1. INTRODUCTION

Milk and its ingredients are often used for food products such as beverages, particularly for coffee or tea. Canned coffee or tea with milk normally includes two types of emulsifiers; one is for bacteriostatic effects and the other is for stability-enhancing effects (Matsumiya, Takahashi, Inoue & Matsumura, 2010). Since bacteriostatic emulsifiers tend to destabilize protein-based emulsions despite of the name "emulsifier" (Matsumiya, Nakanishi & Matsumura, 2011), stability-enhancing emulsifiers are necessary for manufacturing stable products, that is, products with long-term shelf-life.

When both types of emulsifiers are added to canned coffee or tea with milk, the product shows different long-term stability with respect to creaming or aggregation according to different combinations of the emulsifiers. To examine the different long-term stability, manufactures have to spend much time and efforts toward designing optimal formulations in developing new commercial products. In practice, it is quite difficult to predict the stability of the milk-based emulsions during long-term storage and is, therefore, strongly required to develop a simple and rapid method to estimate the long-term stability of the emulsions in short-time period.

An emulsion is thermodynamically unstable and undergoes a time-dependent change of the droplet size distribution based on instability processes such as creaming, flocculation, aggregation and coalescence (Dickinson, 1992). Creaming can be readily confirmed in destabilized emulsions by visual observation and optical methodology, while aggregation of oil droplets can be also detected in macro or micro-ordered scale by visual or microscopic observation and particle size

analysis (McClements, 2005). These analytical techniques, however, cannot be directly applied to the prediction of long-term stability of emulsions in kinetically stable state, i.e., with no creaming or no aggregation in most cases.

In our previous research, the authors investigated potential factors affecting the creaming velocity and aggregation efficiency in milk-based emulsion including bacteriostatic and stability-enhancing emulsifiers; for example, particle size, zeta potential, steric effects of adsorbed layer and so on (Matsumiya *et al*., 2010). The authors clarified that the main factors affecting stability of the milk-based emulsions can be the amount and composition of milk proteins on the oil droplet surfaces. Although their objective for potential factors affecting the destabilization process was, therefore, attained in the previous research, the development of a practical tool for predicting emulsion stability is still required.

Recently, many researchers have been used an optical technique, Turbiscan for evaluation of the stability of emulsions such as creaming or flocculation, which is originally developed by Mengual, Meunier, Cayré, Puech & Snabreb (1999a, b). Juliano, Kutter, Cheng, Swiergon Mawson & Augustin (2011) analyzed creaming of fat globules in raw and recombined milk by Turbiscan, and reported that creaming was more evident in raw milk than that of recombined one. Grotenhuis, Tuinier & Kruif (2003) applied Turbiscan method to concentrated dairy products that can be models of whipped cream or evaporated milk to evaluate a kind of colloidal attractive force, depletion interactions. This method was also utilized for measuring sedimentation velocity of destabilized milk proteins in acidified milk beverages (Laurent & Boulenguer, 2003; Sedlmeyer, Brack, Rademacher & Kulozik, 2004). The Turbiscan techniques are common and advantageous to rapid measurements, and widely applied for food emulsions consisting of milk or its ingredients.

In previous research, the authors performed a centrifugal process on the milk-based emulsions in order to obtain the cream layer for subsequent analyses (Matsumiya *et al*., 2010). They

found a tendency at that process that the collected cream layer, i.e., concentrated oil droplets after centrifugation treatments varied with the formulation of the milk-based emulsions; that is, the more unstable an emulsion was, the more tightly packed and aggregated oil droplets were. This implies some relationship between the aggregation strength of oil droplets and the long-term stability in the milk-based emulsions.

In this research, the author proposes a novel method for rapid determination of aggregation forces between colloidal particles that is developed based on this previous work. The central idea of the novel method is that particles with stronger aggregation forces tend to form aggregates and should not be redispersed easily. The objective of this work is to test the usefulness of our newly developed method as a tool for predicting the long-term stability of emulsions in short-time period, especially, in comparison with Turbiscan method that is common and widely accepted in the fields of food science and food industry.

2. MATERIALS AND METHODS

2.1 Materials

Deionized water was used for the preparation of all the solutions. Corn oil was obtained from Nacalai Tesuque, Inc. (Kyoto, Japan). Fatty-acid free bovine serum albumin (BSA) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Polystyrene latex suspension with particle diameter of 0.5 μm (Polybead polystyrene microspheres, 2.5% solids-latex) was made by Polysciences, Inc. (Pennsylvania, the USA). Powdered milk was obtained from Snow Brand Milk Products Co., Ltd. (Tokyo, Japan). Emulsifiers, P-1670 (sucrose palmitate, HLB 16), S-570 (sucrose stearate, HLB 5) and S-770 (sucrose stearate, HLB 7) were manufactured by Mitsubishi-Kagaku Foods Corporation. Q-14Y (decaglycerol esters of myristic acid, HLB 14.5) was produced by Taiyo Kagaku Co., Ltd. TRP-97RF (triglycerol esters of palmitic acid, HLB 10), DP-95 (diglycerol esters of palmitic acid, HLB 8) and BS-20 (succinate mono-glyceride, HLB 5.5)

were manufactured by Riken Vitamin Co., Ltd. Of these emulsifiers, P-1670, DP-95 and TRP-97RF are with bacteriostatic effects. Powdered milk and all the emulsifiers were stored in a refrigerator prior to use. All other general chemicals used were of analytical grade purchased from Nacalai Tesuque, Inc. (Kyoto, Japan) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Development of a novel method

2.2.1. Preparation of model emulsions and suspensions

Protein solution was prepared by dissolving BSA into 200 mM sodium phosphate buffer (pH 7.0). Stock emulsion was prepared by homogenizing corn oil and the protein solution with a high-speed blender at 20 ºC at 14,000 rpm for 3 minutes (Physcotron, NS-51, Microtec Co., Ltd., Japan). The contents of oil, protein and buffer in the stock emulsion were 2.5, 0.5 and 97.0 wt% respectively. The stock emulsion and the purchased polystyrene latex suspension were mixed with various concentrations of NaCl solutions at the volume ration of 1:4 to prepare model emulsions and model suspensions, respectively. The final concentration of NaCl in these model emulsions and suspensions was from 0 to 400 mM. The model emulsions were heated at 90 ºC in a water bath for 30 min to promote emulsion destabilization. They were cooled down in trash ice and then kept at room temperature for a while. Both the model emulsions and suspensions were transferred into a plastic sample tube with a volume of 1.5 ml.

2.2.2. Forcible aggregation

Colloidal particles in the model emulsions and suspensions were forced to form aggregates through centrifugal treatments. The model emulsions in the sample tube were centrifuged using an ultracentrifuge (CS120, Hitachi Koki Co., Ltd., Japan) at 20 ºC at 140,000g for 20 min. The model suspensions in the sample tube were centrifuged at 20 ºC at 1,960g for 5 min and then centrifuged at 7830g for 10 min using a centrifuge (MR-150, Tomy Seiko Co., Ltd., Japan). This two-step treatment was aimed at collecting the aggregates in the tightly packed state into the bottom part of

the tubes. The two-step treatments were enough to make the colloidal particles thoroughly aggregated.

2.2.3. Redispersion

As a preliminary examination, the centrifuged model emulsions and suspensions were subjected to shaking process by a vortex-type mixer (Vortex genie 2, Scientific Industries Inc., USA) at room temperature at a shaking level of 6, which is just an indication of the mixing speed, in order to redisperse the forcibly aggregated colloidal particles.

As a more sophisticated method controlling the power of redispersion, the following process was employed. The tubes containing the centrifuged model emulsions and suspensions were placed into a tube rack with no bottom and placed on the vibrating part of an active contact speaker (GY-1, Fostex Company, a division of Foster Electric Co., Ltd., Japan) with the top gently pressed by a weight (100 g), and subsequently vibrated to redisperse the forcibly aggregated colloidal particles by the speaker at 100 Hz for various times in an incubator at 20 ºC.

2.2.4. Turbidimetry

After the redispersion treatment, the middle part of the capped sample tube was bored with a flame-heated sharp needle followed by insertion of another injection needle of a syringe. The redispersed layer of the both model dispersions was carefully taken out by the needle-attached syringe with the cap open, and then transferred into another sample tube. The obtained redispersed layer of the model emulsions and suspensions was twice and four-fold diluted with deionized water, respectively, and then subjected to turbidimetric measurements at the wavelength of 600 nm (UV2400-PC, Shimadzu, Japan).

Table 4-1. Compositions of the milk-based emulsions.

Composition (g/L)	1	2	3	4	5	6	7	8
Powdered milk	210.00	210.00	210.00	210.00	210.00	210.00	210.00	210.00
DP-95[*]	3.75			4.50	6.87	6.87		
P-1670[*]		8.01	4.50	4.50			8.01	
TRP-97RF[*]								5.75
BS-20[†]	2.25	6.23			3.44	4.58	4.58	4.58
S-770[†]			3.00	3.00				
S-570[†]					3.44			
Q-14Y[†]						2.75	2.75	2.75

Powdered milk and two kinds of emulsifiers (*: bacteriostatic emulsifiers and †: stability -enhancing emulsifiers) were dispersed in deionized water at the shown weight-per-volume ratio.

2.3. Application of the novel method to milk-based emulsions

2.3.1. Preparation of milk-based emulsions

Compositions of the emulsions used in this research are shown in Table 4-1. Powdered milk-based emulsions were prepared using the following procedure. Emulsifier dispersions were prepared by homogenizing the emulsifiers in the appropriate amount of boiling water using a high-speed blender (Physcotron, NS-51, Microtec Co., Ltd.) at 19,300 rpm for 2 min. Powdered milk was dispersed into the appropriate amount of boiling water using the blender at 19,300 rpm for 2 min, and the resultant milk-based emulsions were then blended with the emulsifier dispersions at 19,300 rpm for 3 min using the blender. The powdered milk-based emulsions were cooled down in trash ice for 3 min, and then filled up with deionized water to adjust the final concentration of powdered milk and the emulsifiers. The resulting milk-based emulsions were autoclaved at 121 ºC for 30 min for sterilization.

2.3.2. Long-term storage stability test

Long-term storage stability of the milk-based emulsions with coffee extract was tested in canned states, which were placed in a practical auto-vending machine set at cold temperature. The

canned milk-based emulsions with coffee extract were sampled after more than one month storage (41, 46 and 51 days; n=3) to evaluate their stability. The cans containing the emulsions were dropped down from the auto-vending machine and then gently opened. The stability of the milk-based emulsions were evaluated by measuring area of white aggregates at the top of liquid surface and reported as surface occupations (area-weighted %). Since the author used large-sized high-resolution photographs as the analysis dataset, the covered area with white aggregates was easily distinguished by their appearance, and can be calculated by manually surrounding the each aggregate by a software, DataPicker ver.1.2 (shareware developed and distributed by Dr. Mizuho Aotsuka, Japan).

2.3.3. Rapid assays

2.3.3.1 Turbiscan

The milk-based emulsions were analyzed by an optical analyzer, Turbiscan (Turbiscan MA 2000, Formulaction, France), developed by Mengual *et al* (1999a). The milk-based emulsions were decanted into a glass cylindrical cell (sample height = 60 mm) and then periodically scanned by the detection head of the device with a pulsed near infrared light source (wave length = 850 nm) from 0 h to 20 h at intervals of 10 min at ambient temperature (20 ℃). The backscattered profiles in a middle part of the emulsions (20 - 50 mm height) were collected as raw data. The mean values of the delta backscattering intensity (changes from 0 h) were reported. The changing rate in the middle part depends on an increased concentration of oil droplets such as flocculation phenomena (Blijdenstein, Hendriks, Van der Linden, Van Vliet & Van Aken, 2003; Silletti, Vingerhoeds, Norde & Van Aken, 2007).

2.3.3.2 Vibration method

The milk-based emulsions were centrifuged using an ultracentrifuge (CS120, Hitachi Koki Co., Ltd., Japan) at 20 ℃ at 140,000g for 20 min to make emulsion oil droplets aggregated. After

this forcible aggregation process, the milk-based emulsions were separated into cream layer containing oil droplets at to the top, clear water phase in the middle and brown precipitate at the bottom of the tube. The forcibly aggregated oil droplets were redispersed using the contact speaker for 90 min in an incubator at 20 ºC according to the procedure which is described above, section 2.2.3. The brown precipitate was so tightly packed that it scarcely contributed to an increase of turbidity of the water phase in this experiment. The redispersed phase was collected by a syringe as described above. It was then twice diluted and underwent turbidimetric analysis.

2.3.4. Statistical analysis

One-way ANOVA was employed to show significant differences between the sample groups with various emulsifier compositions, varied NaCl concentrations or different treatment times Statistical linear regression analysis was applied to the eight compositions of the milk-based emulsions to validate the rapid assays as a method for prediction of the long-term stability of the emulsions. All the statistical analyses were performed using Microsoft Excel 2010 for Windows.

3. RESULTS AND DISCUSSION

3.1. Evaluation of aggregation forces between colloidal particles in model dispersions

3.1.1 Preliminary shaking test by a common device

Aggregation of colloidal particles including flocculation is the process where two or more particles associate with each other. Its rate depends on collision frequency and collision efficiency of dispersed particles (Evans & Wennerstrom, 1999). Main factors affecting the frequency and the efficiency in various physical states are summarized and discussed by McClements (2005). On the other hand, there have been few attempts on evaluating aggregation forces "as a whole" that work between colloidal particles in a dispersion system. As described in the introduction part, there is a possibility that the phenomenon found in our previous research could be developed to an evaluation method for "whole" aggregation forces and utilized as a tool for the prediction of long-term stability

of the milk-based emulsions. In this context, the author was motivated to establish a novel method to determine whole aggregation forces between colloidal particles.

It is widely accepted for researchers in the colloidal science field that electrolytes like ions efficiently screen electrostatic repulsive force between colloidal particles in an emulsion or suspension (McClements, Decker, Park & Weiss, 2008; Sarkar, Goh & Singh, 2009; Singh, Ye & Horne, 2009) and often promotes destabilization of the dispersions such as aggregation (Ise & Sogami, 2005). In the present research, the author prepared soybean oil emulsions stabilized by BSA and polystyrene latex suspension including various concentrations of NaCl as model dispersions with different stability to aggregation. The final concentration of NaCl in the model emulsions and model dispersions was set to 0, 50, 100, 200 or 400 mM and 0, 100, 200 or 400 mM, respectively. The model emulsions were heated at 90 ºC in a water bath for 30 min to promote emulsion destabilization, as BSA adsorbed to oil droplets is denatured at above 67 ºC and exposes its hydrophobic region to aqueous phase, inducing thermal aggregation (Deman, J. M., 1999; Yohannes, G., Wiedmer, S. K., Elomaa, M., Jussila, M., Aseyev, V. & Riekkola, M. L., 2010).

The central idea of the novel method is, again, that particles with stronger aggregation forces tend to form aggregates and should not be redispersed easily. As a preliminary examination, we centrifuged model emulsions and model suspensions to make aggregates, and then shook them by a common shaker, vortex-type mixer to redisperse the aggregates. The author expected that the degree of turbidity is higher for the emulsions and suspensions in the absence of salts as compared to the case that these dispersions include NaCl.

Higher apparent turbidity in redispersed layer was observed for both the model emulsion and dispersion including 100 mM NaCl than no salt, and the increase of the turbidity seemed to depend on shaking treatment times (data not shown). These results suggest that our idea is suitable for evaluating aggregation forces between colloidal particles. Here, the author named this primitive

method "shaking-redispersion method", where the first word is a process for redispersion connected to the key process of the novel method, "redispersion". However, the method we employed above involves ambiguity of redispersion treatments due to the shaker's specs and seems to apply too strong agitation effects for the milk-based emulsions. The author improve the redispersion process by using a more accurately controlled equipment with milder mechanical effects and establish the method by model dispersions whose particles have various aggregation forces between them in the next section.

3.1.2 Testing the novel vibration method on model emulsions and suspensions

In this section, the author tested the novel redispersion method on the model emulsions and suspensions, and examined optimum treatment time for the redispersion process. The author call the method used in this section "vibration-redispersion method". Fig. 4-1a and 4-1b show the turbidity of redispersed aqueous phase of the model emulsions and suspensions containing various concentrations of NaCl after they were subjected to vibration treatments for 60 min. For both the model emulsions and suspensions, the turbidity was significantly different among the five or four NaCl concentrations, respectively (one-way ANOVA, $p < 0.05$). The turbidity of redispersed layer of the emulsions gradually decreased with the increase of ionic strengths between 0 mM and 400 mM (Fig. 4-1a). For the model suspensions, the turbidity linearly decreased depending on the increased NaCl concentrations (Fig. 4-1b). These results indicate that our newly developed method successfully detected the increased aggregation of particles in the model emulsions and suspensions which were mainly caused by the screening effects of the electrolytes. In other words, the method should be valid for the evaluation of aggregation forces.
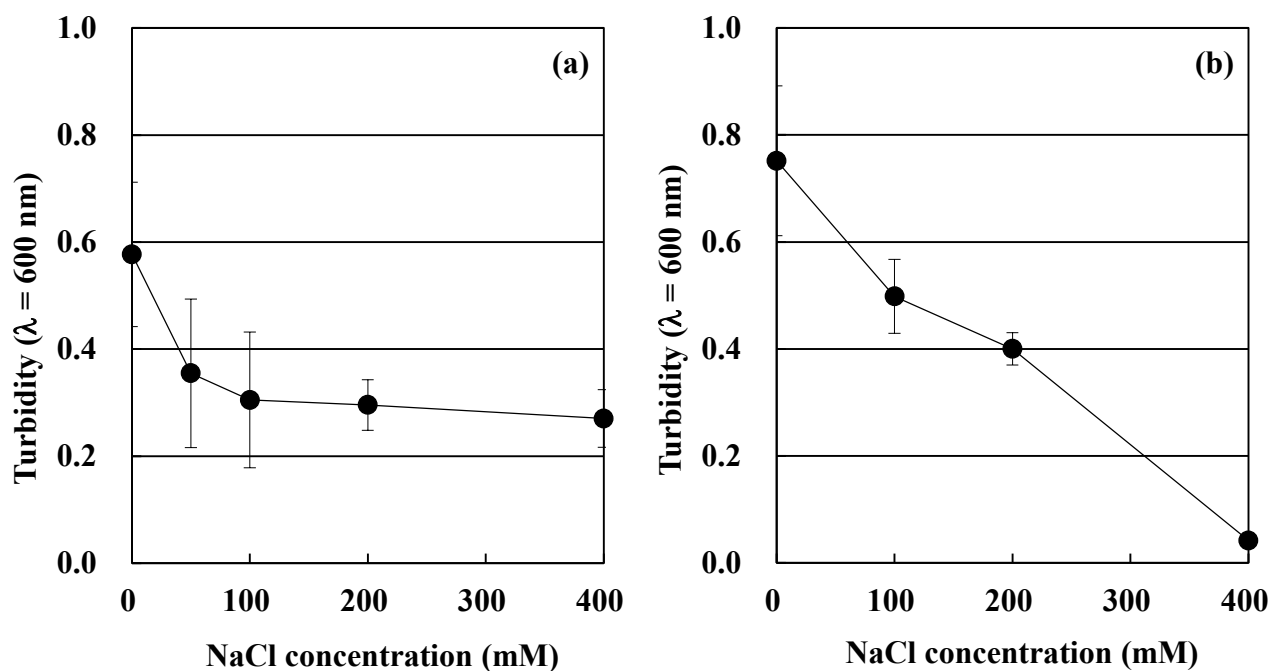
Figure 4-1. Turbidity of redispersed aqueous phase of the model emulsions (a) and suspensions (b) including various concentrations of NaCl.

The turbid layer obtained from the model dispersions was appropriately diluted for spectrophotometric measurements as mentioned in the materials and methods part. Data was represented as mean values ± S.D. (n = 3).

The author examined redispersion processing time that is required for making the difference reflecting the aggregation forces between colloidal particles. Redispersed layer of the model dispersions with no salt or 100 mM NaCl underwent turbidimetric measurements 0, 20, 40 and 60 min after the redispersion treatment by the contact speaker. Fig 4-2a and 4-2b shows time-dependent changes of the turbidity of the model emulsions and the model suspensions. Difference reflecting the order of aggregation stability was observed for the emulsions 20 min or more after the treatment (Fig. 4-2a), while the differences were observed for the suspensions 60 min after the treatment (Fig. 4-2b). These data suggest that 60 min or longer time is at least required for the redispersion process in order to evaluate other kinds of dispersions. There is a possibility that we should modify processing times depending on the nature or characteristics of each sample.
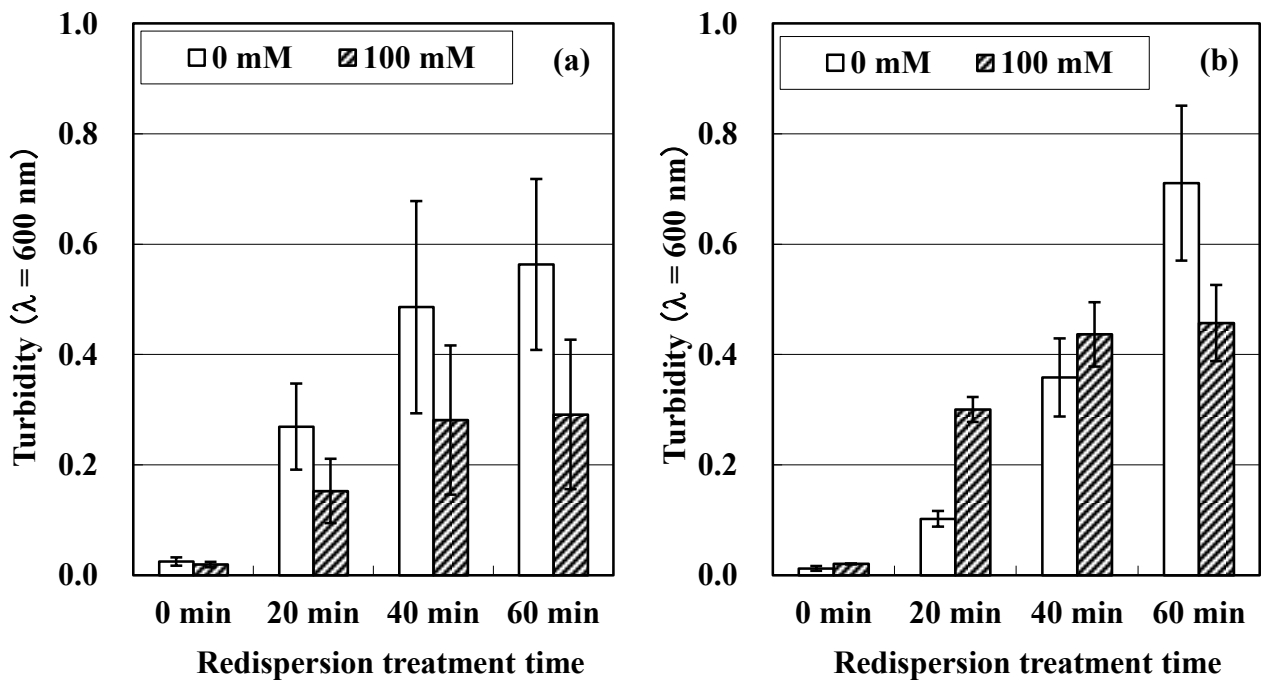
Figure 4-2. Time-dependent changes of the turbidity of redispersed aqueous phase obtained from the model emulsions (a) and suspensions (b) including no salt or 100 mM NaCl.

The turbid layer obtained from the model dispersions was appropriately diluted for spectrophotometric measurements as mentioned in the materials and methods part. Data was represented as mean values ± S.D. (n = 3).

3.2. Application of the novel method to milk-based emulsions

3.2.1. Evaluation of long-term stability of milk-based emulsions

In order to test the long-term stability of the milk-based emulsions including various compositions of emulsifiers, the emulsions were stored in a practical auto-vending machine set at cold temperature more than one month (41, 46 and 51 days; n = 3). It is empirically known that in this commercial condition, fat globules in the milk-based emulsions are destabilized to form aggregates after 30 days or less storage, and the destabilized states do not considerably change during further storage from 40 days to expiration date.

Fig. 4-3 shows the appearance of the milk-based emulsions after 45 days of storage and surface occupations of white aggregates at the top of the samples. The author defined the

| 1 | 0.72 ± 0.45 (%) | 2 | 14.5 ± 6.39 (%) | 3 | 6.76 ± 2.37 (%) | 4 | 38.42 ± 6.01 (%) |
| 5 | 9.18 ± 4.77 (%) | 6 | 17.01 ± 8.9 (%) | 7 | 22.53 ± 3.67 (%) | 8 | 11.03 ± 6.32 (%) |
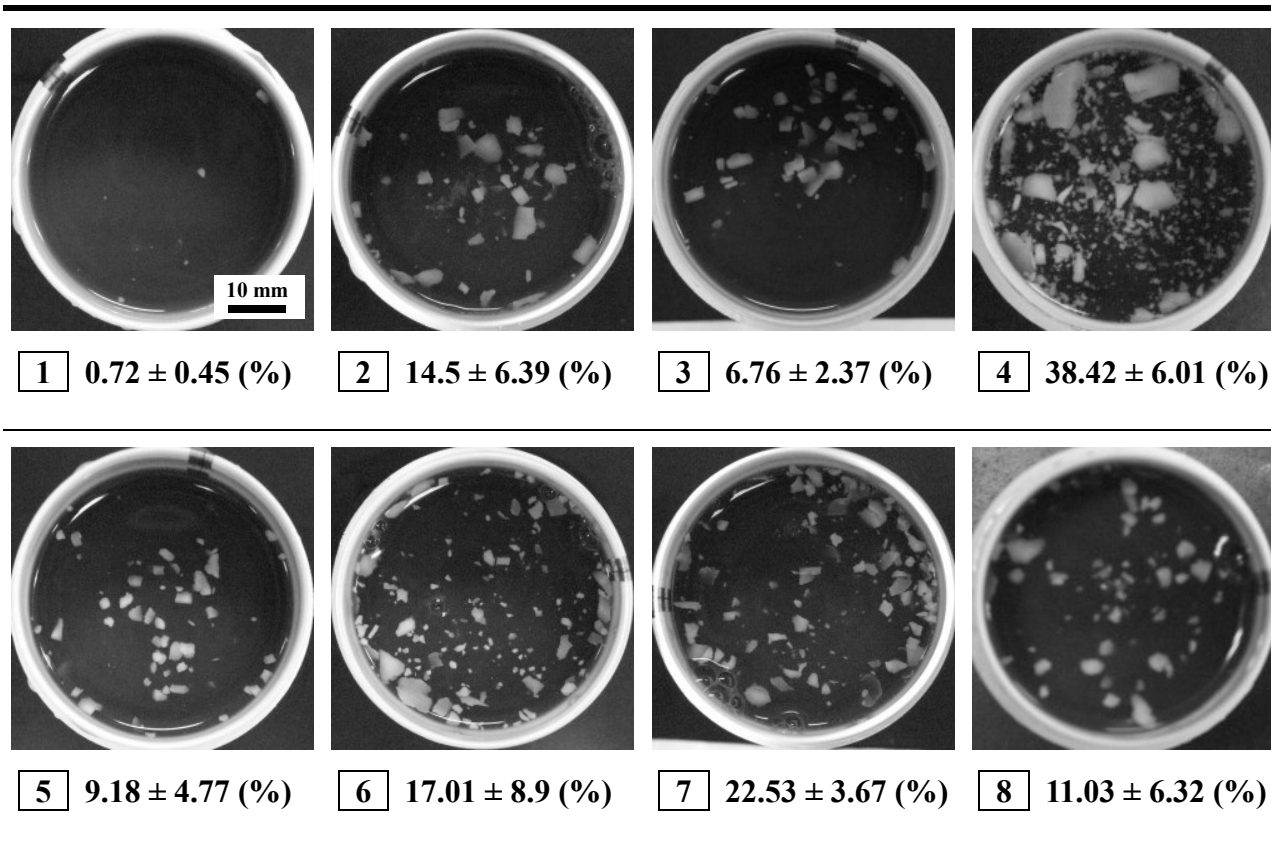
Figure 4-3. Milk-based emulsion with coffee extract including various compositions of emulsifiers (Compositions 1- 8) stored in a practical auto-vending machine set at cold temperature for 45 days.

Values are mean values ± S.D. of surface occupations (area-weighted %) of white aggregates at the top of liquid surface that were measured after 41, 45 and 51 day-storages.

occupation as an index of the stability of emulsions. The stability of the emulsions represented as surface occupation varied according to compositions of the emulsifiers (one-way ANOVA, $p < 0.05$). Composition 1 was the most stable with few aggregates, while Composition 4 was the most unstable with many aggregates. The author tried to find a relationship between the emulsion stability and compositions of the emulsifiers to predict the stability based on the formulation, but it was difficult to find a clear relationship (Figure 4-3 and Table 4-1).

The following experiments were carried out to examine whether or not the two kinds of rapid assays evaluating emulsion stability can be applied to the prediction of long-term stability of the milk-based emulsions. The milk-based emulsions without coffee extract were subjected to the
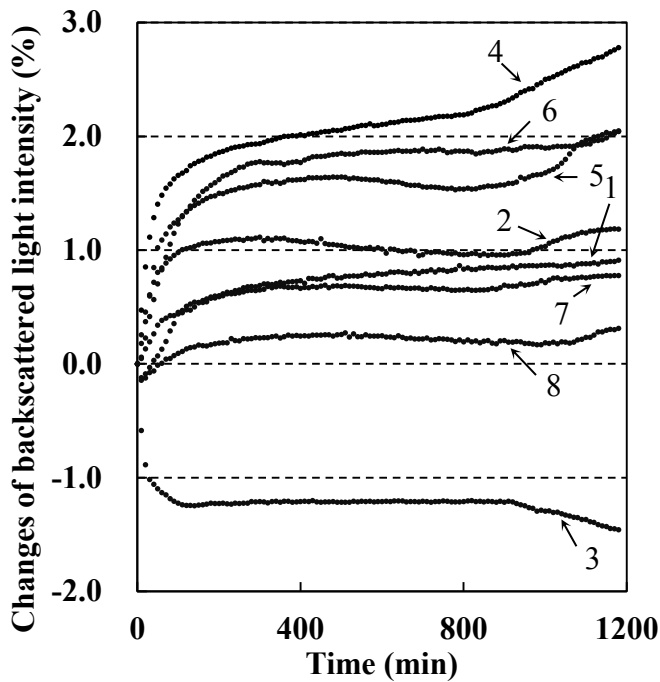
Figure 4-4. Time-dependent changes of backscattered light intensity from the milk-based emulsions with various compositions of the emulsifiers.

The periodical change was monitored in the middle part of the container by Turbiscan equipment for about 20 h (1180 min). The numbers in the figure show Compositions 1-8, respectively.

rapid assays because coffee extract can affect the results of optical measurements used in our method and Turbiscan.

3.2.2. Evaluation of milk-based emulsions by rapid assays

The author measured changes of backscattered light intensity from the milk-based emulsions in the middle part of the glass container to analyze the initial aggregation or flocculation process for about 20 h (1180 min) which could reflect the long-term stability of the milk-based emulsions by Turbiscan equipment. Figure 4-4 shows periodical changes of the backscattered light intensity from the emulsions with various compositions of the emulsifiers. For all of the samples except Composition 3, increased changes of the light intensity were observed during the monitoring process. This result indicates that in most cases the concentration of oil droplets present in the middle part of the emulsions increased in time-dependent manner probably due to the flocculation of droplets (Blijdenstein *et al.*, 2003; Silletti *et al.*, 2007). The decreased backscattered light intensity from the Composition 3 emulsion can be attributed to the creaming of the fat globules to the top or precipitation of the milk protein particles toward the bottom.
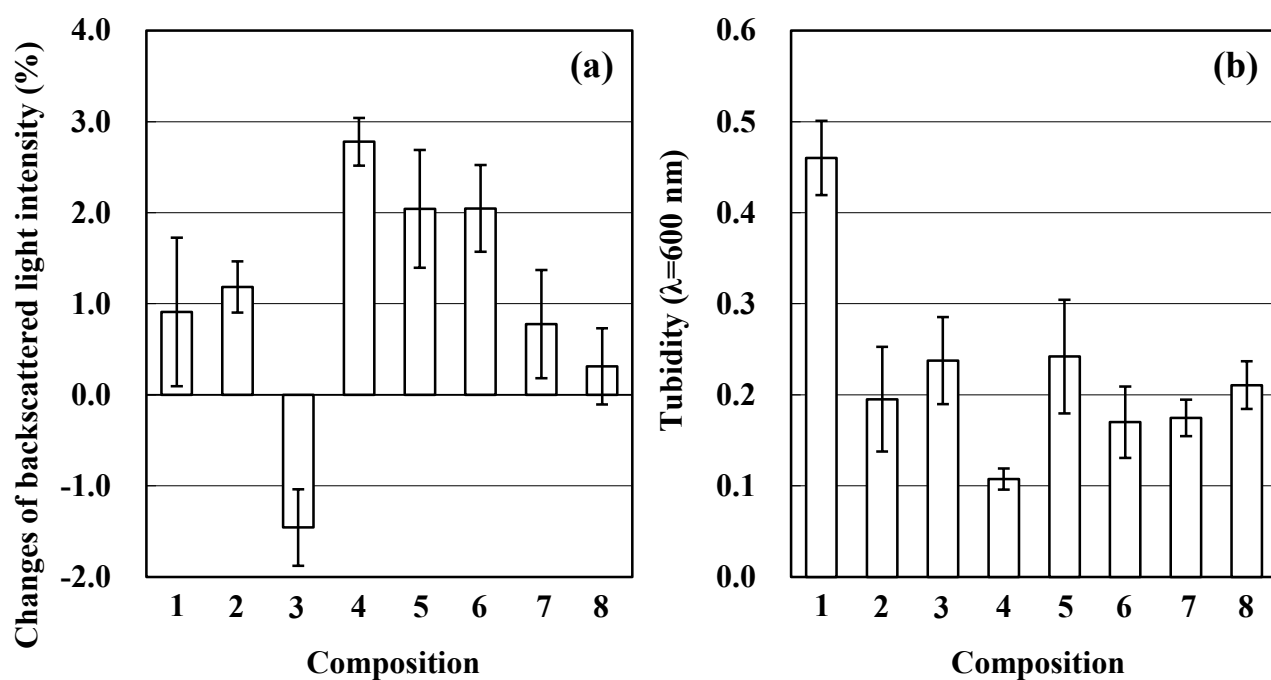
Figure 4-5. The changed backscattered light intensity of the milk-based emulsions at 1180 min (a) and the turbidity of the appropriately diluted continuous phase of the milk-based emulsions redispersed for 90 min (b).

Data was represented as mean values ± S.D. (n = 3).

Although both concentration of oil droplets and particle size changes by flocculation observed as the changed backscattered light intensity in the initial stage of long-term storage might be, in principle, completely different from oil droplet aggregation in long-term storage, oil droplets tend to aggregate according to the higher collision efficacy depending on the increased concentration of oil droplets and then aggregate with each other normally through flocculated state. On the basis of this idea, the author applied the data obtained by Turbiscan equipment to the prediction of long-term stability of the milk-based emulsions. The author employed the changed backscattered light intensity at 1180 min with the most appreciable differences between the light intensity. The employed data representing mean values with standard deviations (n = 3) are shown in Figure 4-5a. The same formulations of the milk-based emulsions were also subjected to our newly developed method, vibration-redispersion method. The turbidity of the continuous layer of
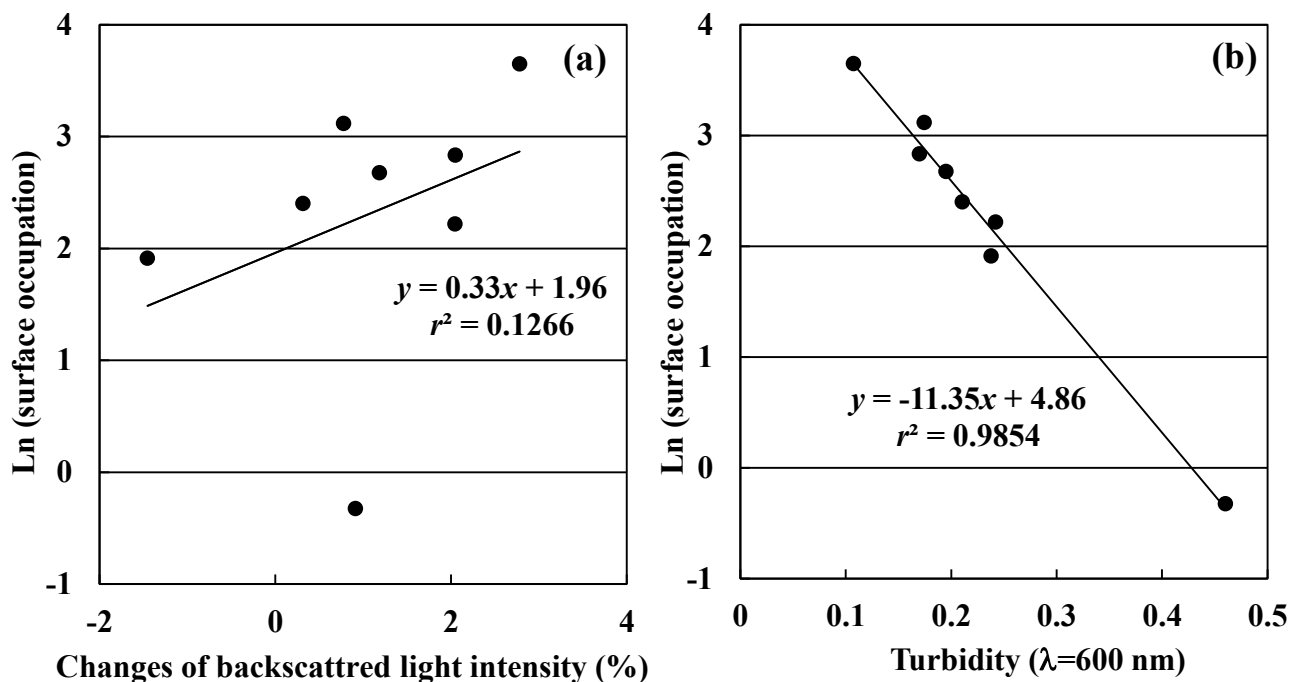
Figure 4-6. Relationships between the long-term stability of the milk-based emulsions and the initial aggregating process evaluated by Turbiscan method (a) or the aggregation forces evaluated the vibration-redispersion method (b). Linear regression analysis was performed on the different datasets. Regression equations with contribution ratios (r^2) were also demonstrated in the figures. Data plots were represented as mean values (n = 3).

the milk-based emulsions redispersed for 90 min was demonstrated in Figure 5b. Both the changed backscattered light intensity (Figure 4-5a) and the turbidity (Figure 4-5b) were significantly different according to the emulsifier compositions (one-way ANOVA, $p < 0.05$), suggesting that the data obtained by the two rapid assays can be utilized as different datasets for the prediction of long-term stability of the milk-based emulsions.

3.2.3. Validation of the novel method by linear regression

In order to validate the rapid assays, the author performed statistical regression analyses on the datasets evaluated by the rapid assays and long-term storage stability test. They were more properly fitted to a linear function using a log-linear regression model than a standard linear regression model particularly for the vibration-redispersion method (Figure 4-6b). The author describes a possibility that long-term stability of the milk-based emulsions follows a power law for

the surface occupation of white aggregates with aggregation force represented as the turbidity of redispersed layer. The data clearly shows that the long-term stability of the emulsions can be better predicted by the aggregation forces evaluated the vibration-redispersion method (Figure 4-6b) than the initial aggregating process evaluated by Turbiscan method (Figure 4-6a). The author could have found a good correlation between the stability of coffee and our novel method in this context. However, whether this method is generally applicable or not should be further examined elsewhere by calibrating the evaluation system in each food dispersion system.

In this work, we adopted an unstable system where relatively strong aggregation of oil droplets was caused by addition of electrolytes in order to establish our novel method. The next step of the research is to examine validity of the method to emulsions stabilized by various mechanisms particularly with weaker aggregation forces such as depletion flocculation, bridging flocculation or partial coalescence. Such model emulsions can be constructed by using emulsions with varied concentrations of hydrocolloids, with bridging polysaccharides like pectin, or with partial-coalescence promoting emulsifiers including lecithin under low temperatures, respectively.

4. CONCLUSION

The author established a novel method for rapid determination of aggregation forces between colloidal particles and successfully applied the method to the prediction of long-term stability of milk-based emulsions including various compositions of emulsifiers. A graphical procedure of the method is demonstrated in Figure 4-7. The author named the novel method shaking-redispersion method or vibration-redispersion method. In principle, the author can develop ultrasonication-redispersion method, stirring-redispersion method and other types of method according to the redispersion process. The author can point out a future possibility that the method is applicable for the evaluation of potential ability of gel formations or other phenomena that are based on the aggregation process in addition to the prediction of emulsion or suspension stability.
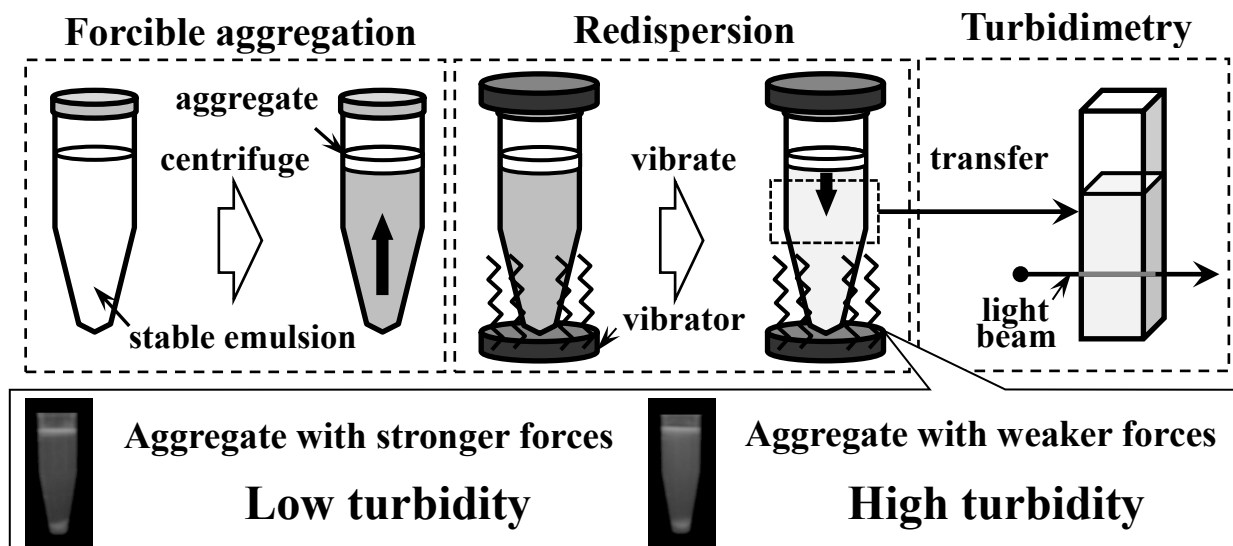
Figure 4-7. Graphical procedure of the newly developed vibration-redispersion method for evaluating aggregation forces between oil droplets in an emulsion.

Finely dispersed oil droplets are forced to form aggregates through centrifugal treatments. The centrifuged model emulsions and suspensions were subjected to vibration process by a contact speaker in order to redisperse the forcibly aggregated colloidal particles. After the redispersion treatment, the middle part of the sample was then subjected to turbidimetric measurements.

REFERENCES

Blijdenstein, T. B. J., Hendriks, W. P. G., van der Linden, E., van Vliet, T. & van Aken, G. A. (2003). Control of strength and stability of emulsion gels by a combination of long- and short-range interactions. *Langmuir* 19, 6657-6663.

Deman, J. M. (1999). *Principles of food chemistry*, third edition, Springer Science+Business Media, NY, USA. pp. 111-162.

Dickinson, E. (1992). *An introduction to food colloids*, Oxford, UK: Oxford University Press. pp. 79-122.

Evans, D. F. & Wennerström, H. (1999). *The Colloidal Domain: Where Physics, Chemistry, Biology, and Technology Meet*, second edition. Wiley-VCH, Weinheim, Germany. pp. 401-442.

Grotenhuis, E., Tuinier, R. & Kruif, C. G. (2003). Phase stability of concentrated dairy products. *Journal of Dairy Science* 86, 764-769.

Ise, N., & Sogami, I (2005). *Structure formation in solution: Ionic polymers and colloidal particles*, Springer-Verlag, Heidelberg, Germany. pp. 1-30.

Juliano, P., Kutter, A., Cheng, L.J., Swiergon P., Mawson, R. & Augustin, M.A. (2011). Enhanced creaming of milk fat globules in milk emulsions by the application of ultrasound and detection by means of optical methods. *Ultrasonics Sonochemistry* 18, 963–973.

Matsumiya, K., Takahashi, W., Inoue, T., & Matsumura, Y. (2010). Effects of bacteriostatic emulsifiers on stability of milk-based emulsions. *Journal of Food Engineering* 96, 185-191.

Matsumiya, K., Nakanishi, K. & Matsumura, Y. (2011). Destabilization of protein-based emulsions by diglycerol esters of fatty acids -The importance of chain length similarity between dispersed oil molecules and fatty acid residues of the emulsifier, *Food Hydrocolloids* 25, 773-780.

McClements, D. J. (2005). *Food Emulsions: Principles, practice, and techniques*, second edition, Boca Raton, Florida, CRC Press. pp. 269-339.

McClements, D., Decker, E., Park, Y. & Weiss, J. (2008). Designing food structure to control stability, digestion, release and absorption of lipophilic food components. *Food Biophysics* 3(2), 219–228.

Mengual, O., Meunier, G., Cayré, I., Puech, K. & Snabre, P. (1999a). Characterisation of instability of concentrated dispersions by a new optical analyser: the TURBISCANMA1000. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 152, 111–123.

Mengual, O., Meunier, G., Cayré, I., Puech, K. & Snabre, P. (1999b). TURBISCAN MA 2000: Multiple light scattering measurement for concentrated emulsion and suspension instability analysis. *Talanta* 50, 445-456.

Sarkar, A., Goh, K. K. T. & Singh, H. (2009). Colloidal stability and interactions of milk-protein-stabilized emulsions in an artificial saliva. *Food Hydrocolloids* 23(5), 1270–1278.

Sillettia, E., Vingerhoeds, M. H., Norde, W. & Van Aken, G. A. (2007). The role of electrostatics in saliva-induced emulsion flocculation. *Food Hydrocolloids* 21, 596-606.

Sedlmeyer, F., Brack, M., Rademacher, B. & Kulozik, U. (2004). Effect of protein composition and homogenisation on the stability of acidified milk drinks. *International Dairy Journal* 14, 331–336.

Singh, H., Ye, A. & Horne, D. (2009). Structuring food emulsions in the gastrointestinal tract to modify lipid digestion. *Progress in Lipid Research* 48(2), 92–100.

Laurent, M.A., & Boulenguer, P. (2003). Stabilization mechanism of acid dairy drinks (ADD) induced by pectin. *Food Hydrocolloids* 17(4), 445–454.

Yohannes, G., Wiedmer, S. K., Elomaa, M., Jussila, M., Aseyev, V. & Riekkola, M. L. (2010).

Thermal aggregation of bovine serum albumin studied by asymmetrical flow field-flow fractionation. *Analytica Chimica Acta* 675, 191–198.

SUMMARY

Chapter 1:

Effects of bacteriostatic emulsifiers on stability of milk-based emulsions

For milk-based emulsion products such as canned coffee or tea, the addition of bacteriostatic emulsifiers is necessary to inhibiting the growth of heat-resistant sporeformers. Since bacteriostatic emulsifiers often cause the destabilization of emulsions, other type of emulsifiers, such as stability-enhancing ones, are necessary for the long-term stability of emulsions. Four milk-based emulsions were prepared from powdered milk combined with several types of emulsifiers. The long-term stability of emulsions, which was detected by the occurrence of a creaming layer after 3 months of storage, differed according to the composition of emulsifiers. To understand the reason for the differences in the stability of emulsions, particle size, distribution, ζ-potential, and the amount of proteins and phospholipids present in the cream layer (separated oil droplets) in the emulsions were measured. Only the amount of proteins adsorbed onto oil droplets was found to be closely related to the difference in emulsion stability, that is, the more proteins adsorbed, the higher the emulsion stability. SDS-PAGE analyses revealed that κ-casein and β-lactoglobulin play an important role in emulsion stability by adsorbing onto the oil droplet surface.

Chapter 2:

Destabilization of protein-based emulsions by diglycerol esters of fatty acids -The importance of chain length similarity between dispersed oil molecules and fatty acid residues of the emulsifier

Destabilizing effects of diglycerol esters of different mono-saturated or unsaturated fatty acids (DF)

on protein-based emulsions prepared with various types of oil were examined by visual observations and particle size analyses. By diglycerol esters of mono-oleic acid (DO), hydrocarbon emulsions were more obviously destabilized than food oil emulsions. Interfacial tension measurements indicated that the adsorbed protein on oil droplet surfaces of hydrocarbon emulsions can be more easily displaced by DO compared to the case of food oil emulsions. The degree of hydrocarbon emulsion destabilization by DO varied with the chain length of hydrocarbon molecules. From the results of combination tests of five hydrocarbons varying in chain length in oil phase and five DF having different mono-fatty acid residue, it was revealed that DF could most effectively destabilize the hydrocarbon emulsion when the chain length of fatty acid residue of DF was similar to that of hydrocarbon molecules.

Chapter 3:

Diglycerol esters of fatty acids promote severe coalescence between protein-stabilized oil droplets by emulsifier-protein competitive interactions

Diglycerol esters of mono-oleic acid (DO), a bacteriostatic emulsifier causes severe coalescence of oil droplets stabilized by milk proteins and phase separation between oil and aqueous phase under agitating conditions. In order to clarify the destabilizing mechanism of the emulsifier, physicochemical and colloidal properties of the emulsifier were compared to similar emulsifiers without destabilizing effects. DO, that is dispersible both in oil and water, adsorbed to the oil-water interface to reduce the interfacial tension, and migrated from oil phase to aqueous phase and vice versa in a plane interface system. Experiments performed in an emulsion system revealed that DO had little ability to emulsify food-grade oil, but displaced milk proteins from the oil droplet surface. These data indicate that DO with little emulsifying ability predominantly occupies the oil droplet

surfaces via emulsifier-protein competitive interactions to promote severe coalescence of emulsion oil droplets probably because DO actively migrates between oil and aqueous phase and it favors to form the planar oil-water interface.

Chapter 4

Evaluation of long-term stability of milk beverages by a novel method for rapid determination of aggregation forces between colloidal particles

Long-term stability of milk-based emulsions in canned coffee or tea varies with the formulation of small-molecule emulsifiers aiming at bacteriostatic effects or stability-enhancing effects. To predict the long-term stability of the emulsions in short-time period, we developed a novel method for rapid determination of aggregation forces between colloidal particles using model emulsions and suspensions with different stability to aggregation for the first step. The novel method was based on an idea that particles with stronger aggregation forces tend to form aggregates and cannot be readily redispersed. While the milk-based emulsions were subjected to long-term storage test with coffee extract, the same emulsions, but not including coffee extract, were rapidly evaluated by both the novel method and a common method, Turbiscan analysis often applied to the evaluation of emulsion stability. Statistical regression analysis according to the datasets obtained by the two rapid assays revealed that the long-term stability of the milk-based emulsions can be better predicted by the aggregation forces evaluated by the newly developed method than the initial aggregating process evaluated by Turbiscan method. The author named the novel method "vibration-redispersion method".

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LIST OF PUBLICATIONS

1. Effects of bacteriostatic emulsifiers on stability of milk-based emulsions.

   Kentaro Matsumiya, Wataru Takahashi, Takashi Inoue and Yasuki Matsumura

   Journal of Food Engineering (2010), 96, 185-191.

2. Destabilization of protein-based emulsions by diglycerol esters of fatty acids - The importance of chain length similarity between dispersed oil molecules and fatty acid residues of the emulsifier.

   Kentaro Matsumiya, Koichi Nakanishi and Yasuki Matsumura

   Food Hydrocolloids (2011), 25, 773-780.

3. Evaluation of long-term stability of milk beverages by a novel method for rapid determination of aggregation forces between colloidal particles.

   Kentaro Matsumiya, Takashi Inoue, Joju Niida, Takao Katagiri, Takahisa Nishizu and Yasuki Matsumura

   Food Hydrocolloids (2014), 34, 177-183.

4. Diglycerol esters of fatty acids promote severe coalescence between protein-stabilized oil droplets by emulsifier-protein competitive interactions.

   Kentaro Matsumiya, Yasuaki Takahashi, Koichi Nakanishi, Nobuo Dotsu and Yasuki Matsumura

   (*In submission*)