Coalescence in protein stabilized emulsions

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ABSTRACT

The coalescence stability of β-lactoglobulin (BLG) and whey protein concentrate (WPC) stabilised, oil-in-water emulsions is studied by centrifugation. The stability tests are complemented with determination of the protein adsorption on the surface of the oil drops. The results show that, in both systems, the coalescence stability increases in a step-wise manner when plotted as a function of the protein adsorption: a well-defined, threshold value of the protein adsorption exists, at which a sharp increase of emulsion stability is observed. This value is slightly lower than the density of the complete monolayer of adsorbed protein molecules. The effects of two other factors, the storage time (i.e., the surface aging) and the thermal treatment, on the emulsion stability are also studied. We found that the emulsion stability of BLG emulsions significantly decreases after one day of shelf-storage at room temperature, as compared to the stability of freshly prepared emulsions. No such effect of surface aging was observed with WPC-stabilized emulsions. The obtained results are discussed by considering the variations in the density and structure of the protein adsorption layers.

1/ Introduction.

In a previous paper (1) we studied how the coalescence stability of emulsions containing β-lactoglobulin (BLG) depends on protein adsorption at the drop surface and on drop size. For this purpose, the emulsion stability was characterized by centrifugation, the protein adsorption was measured by Bradford method, and the drop size-distribution was determined by optical microscopy (1).

For comparison, similar series of experiments was performed in the present study with emulsions stabilized by whey protein concentrate (WPC), whose main protein component is BLG. We studied also the effects of storage time and thermal treatment on the coalescence stability of both BLG and WPC containing emulsions. Interestingly, we found that the emulsion stability of BLG emulsions significantly decreases after one day of storage, as compared to the stability of freshly prepared emulsions (aging effect). No aging effect was observed with WPC-stabilized emulsions. The effect of thermal treatment on the BLG and WPC-containing emulsions was also different – the stability of BLG emulsions increased and the aging effect disappeared, whereas the stability of WPC emulsions remained unaffected. Several experiments, aimed to clarify the reasons for the aging effect and for the differences between BLG and WPC stabilized emulsions were performed. In a parallel study (2), we present a larger set of results from comparative experiments with BLG and WPC emulsions, and analyze in more detail the reasons for the observed differences between these two similar in composition protein systems.

2/ Materials and Methods.

2.1. Materials. The proteins used are β-lactoglobulin, (BLG, product of Sigma) and whey protein concentrate (WPC; trade name AMP 8000; product of Proliant). WPC contains 72 % proteins (the major protein component is BLG, 44 wt % from the total protein content), 14 % carbohydrates, 6 % water, 3 % ash, and 5 % fat. The proteins were used as received. The soybean oil (used as an oil phase) was purified from polar contaminants by passing through a glass column filled with Florisil adsorbent (3). The aqueous solutions were prepared with deionized water, purified by a Milli-Q Organex system (Millipore). Along with the protein, all solutions contained 0.15 M NaCl (Merck, analytical grade) and 0.01 wt % NaN_3 (Riedel-de Haën). The pH was 6.2 (the natural one).

2.2. Emulsion preparation. The oil-in-water emulsions, stabilized by BLG, were prepared by intensive stirring of 35 mL protein solution and 15 mL soybean oil (30 vol. %) with a rotor-stator homogenizer Ultra Turrax T25 (Janke & Kunkel GmbH & Co, IKA-Labortechnik) operating at 13 500 rpm. The duration of stirring was fixed at 3 min. It was shown (1) that this homogenization procedure gives emulsions with very similar drop-size distributions and almost the same average drop diameter, which allowed us to separate the drop-size effect from the other effects on emulsion stability.

The oil-in-water emulsions, stabilised by WPC, were prepared by using a two-step procedure. Initially, a premix of 520 mL protein solution and 200 mL oil (28 vol. %) was prepared by hand-shake homogenisation. The second homogenisation step was accomplished by using a custom-made, narrow-gap emulsifier (2). The duration of the emulsification was fixed at 10 min, which corresponded to 100 cycles (passages) of the emulsion through the emulsification device.

2.3. Determination of drop size distribution. The drop size distribution in the emulsions was determined by optical microscopy. The specimens were taken immediately after emulsion preparation.

The oil drops were observed in transmitted light with microscope Axioplan (Zeiss, Germany), equipped with objective Epiplan ×50, and connected to CCD camera (Sony) and video-recorder (Samsung SV-4000). The diameters of the recorded oil drops were afterwards measured (one by one) with software for image analysis, and the mean volume-surface diameter, d_{32}, was calculated.

2.4. Determination of protein adsorption. The protein adsorption on the drop surface, Γ, was calculated from the specific surface area of the oil drops, $S = 6/d_{32}$, and from the change of the protein concentration in the aqueous phase (the serum) as a result of the emulsification process (1). The protein concentration in the serum was determined by the method of Bradford (4).

2.5. Evaluation of emulsion stability by centrifugation. The emulsions were centrifuged at 20°C in 3K15 centrifuge (Sigma Laborzentrifugen, Germany). We characterize the emulsion stability by the critical osmotic pressure, P_{OSM}^{CR}, at which oil is released at the top of the emulsion cream in the centrifuge tube (1). P_{OSM}^{CR} is calculated from the experimental data, under the assumption that the centrifugal acceleration is homogeneous along the cream, by using the following equation (1):

$$P_{OSM}^{CR} = \Delta\rho \, g_k \left(V_{OIL} - V_{REL} \right) / A \qquad [1]$$

where $\Delta\rho$ is the difference between the mass densities of the oil and the aqueous phase; g_k is the centrifugal acceleration; V_{OIL} is the *total volume of oil* used for preparation of the emulsion; V_{REL} is the volume of *released oil* at the end of centrifugation; and A is the cross-sectional area of the centrifuge test tube. The principle of the method and the used procedures are described in detail in Ref. (1).

3/ Results and discussion.

3.1. Dependence of emulsion stability on protein adsorption. The stability against coalescence of BLG-containing emulsions has been studied in Ref. (1). The obtained results show the presence of a well defined threshold value of the BLG adsorption on the drop surface, $\Gamma^* \approx 1.55$ mg/m^2, which is required for obtaining stable emulsions: the emulsions are very unstable at $\Gamma < \Gamma^*$, a step-wise increase of stability is observed at $\Gamma \approx \Gamma^*$, and a further, much slower increase is observed at $\Gamma > \Gamma^*$ (see Figure 1A). The value of Γ^* is slightly lower than the adsorption value for a dense monolayer of BLG molecules, $\Gamma_M \approx 1.65$ mg/m^2, and corresponds to a threshold surface coverage of $\theta^* = (\Gamma^*/\Gamma_M) \approx 0.9$.

In the present study, we performed a similar series of experiments with WPC emulsions in the concentration range between 0.02 and 0.1 wt % of WPC: the emulsion stability was evaluated by centrifugation and the protein adsorption was determined by the Bradford method, as described in sections 2.4, 2.5 and Ref. (1). The obtained dependence of the critical osmotic pressure P_{OSM}^{CR} on Γ is plotted in Figure 1B. As seen from Figure 1B, there is a large step in the emulsion stability at Γ^* around 1.9 mg/m^2: the stability is rather low when the adsorption is smaller than Γ^*, and a gradual increase of stability is observed at $\Gamma > 2.2$ mg/m^2.

The obtained value of $\Gamma^* \approx 1.9$ mg/m^2 is larger than that for BLG emulsions (≈ 1.55 mg/m^2), but still lower than the adsorption in a complete protein monolayer for WPC emulsions, 2.4 mg/m^2 (2). From the values of Γ^* and Γ_M, one can calculate the threshold value of the relative surface coverage, $\theta^* = (\Gamma^*/\Gamma_M) \approx 0.8$ for WPC emulsions, which is close to the value found for BLG emulsions ($\theta^* \approx 0.9$).
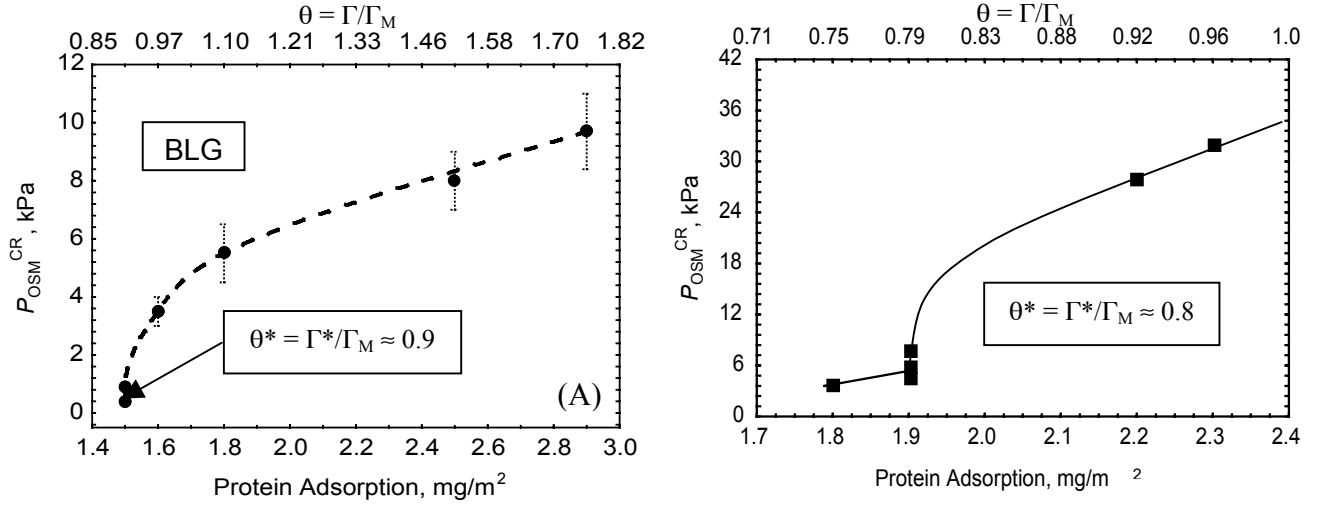
Figure 1. Critical osmotic pressure, P_{OSM}^{CR}, as a function of protein adsorption, Γ, and the relative surface coverage, $\theta = \Gamma/\Gamma_M$, where Γ_M is the density of complete monolayer of adsorbed protein molecules for: (A) BLG and (B) WPC emulsions.

It is worthwhile noting that we were unable to form emulsions containing micrometer-sized drops at WPC concentration smaller than 0.02 wt. %. At such low concentrations, millimeter-sized oil drops were always seen in the emulsion, immediately after its preparation. This result is due to the almost complete exhaustion of the protein solution during emulsification − a simple numerical estimate shows that the amount of protein in the aqueous phase was insufficient to cover the surface of micrometer drops with an adsorption layer of $\theta^* \approx 0.8$. As a result, a significant drop-drop coalescence occurred during and after emulsification. This observation confirmed the result that an almost complete protein layer should be built on the drop surface to obtain a stable emulsion.

The comparison of Figures 1A and 1B shows that the WPC emulsions were more stable (P_{OSM}^{CR} was larger) than the BLG emulsions at similar surface coverage, θ. This difference is most probably related to the different mean size of the oil drops in the two types of emulsion, which were produced by different equipments - Ultra Turrax for BLG and narrow-gap homogenizer for WPC emulsions, respectively (section 2.2). The mean volume-surface diameter in BLG emulsions was 38 μm, whereas that in WPC emulsions was 16 μm. The effect of drop size on emulsion stability was studied in detail in Ref. (1) and it was shown that the value of P_{OSM}^{CR} strongly decreased with the increase of drop size (i.e., the emulsions containing larger drops are less stable at similar surface coverage).

3.2. Effect of storage time on coalescence stability. The emulsion stability against coalescence (quantified by the value of P_{OSM}^{CR}) as a function of the period of emulsion shelf-storage was investigated for BLG (0.02 and 0.1 wt %) and WPC (0.04 and 0.1 wt %) containing emulsions. The following protocol was used: A series of BLG or WPC emulsions was prepared under equivalent conditions. Afterwards, these emulsions were stored undistributed at a room temperature in a gravity field for different periods of time, from 1 min (i.e., immediately after emulsion preparation) up to 14 days. The

critical osmotic pressure for coalescence, P_{OSM}^{CR}, was periodically measured during the storage period by centrifugation. In parallel, the drop size distribution and protein concentration in the serum were determined as functions of the storage time.

The measured critical osmotic pressure remained virtually constant for the WPC emulsions up to 14 days of shelf-storage, that is no aging effect was observed for these emulsions.

In Figure 2A we present the results for P_{OSM}^{CR}, as a function of the storage time for emulsions stabilized by 0.02 wt. % BLG. Three different stages in the emulsion evolution are distinguished: (1) Fast increase of emulsion stability for storage times between 1 and 5 min; (2) A subsequent plateau in the interval between ca. 5 and 180 min; (3) A significant decrease of emulsion stability at longer times of storage (note the logarithmic time-scale in Figure 2).
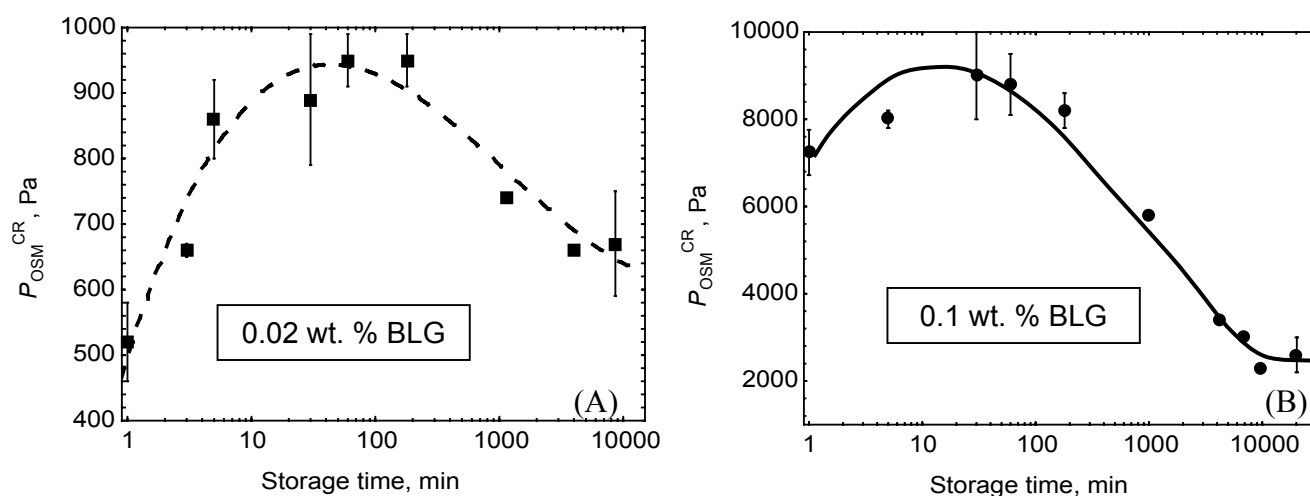

Figure 2. Critical osmotic pressure, P_{OSM}^{CR}, as a function of storage time for: (A) 0.02 wt. % and (B) 0.1 wt. % BLG containing emulsions.

A significant dependence of emulsion stability on storage time was obtained for emulsions stabilized by 0.1 wt % BLG as well, but several important differences (in comparison with the emulsions containing 0.02 wt % BLG) were noticed - see Figure 2B. The first stage, the rapid increase of emulsion stability, is almost missing at the higher protein concentration - the increase is only about 25 % at 0.1 wt % BLG, whereas P_{OSM}^{CR} increases about two times for emulsions stabilized by 0.02 wt. % BLG (cf. Figures 2A and 2B). On the other side, during the third stage of shelf-storage P_{OSM}^{CR} decreases by more than 4 times at the higher protein concentration (0.1 wt %), whereas the decrease is only by about 70 % for the emulsion stabilized by 0.02 wt % BLG. In other words, the first stage (increase of stability) is more pronounced at a lower protein concentration, while the third stage (loss of stability) is much more pronounced at the higher protein concentration. As shown in Ref. (1), an adsorption *monolayer* is formed at 0.02 wt %, whereas an adsorption *bilayer* is formed at 0.1 wt % BLG. Therefore, the aging effect is much more pronounced for the emulsion drops whose surface is covered by a thicker protein layer.

The initial rapid increase of P_{OSM}^{CR} at 0.02 wt % BLG (stage 1) is most probably due to a continuing building of the adsorption layers on the drops' surface during the first several minutes after emulsion preparation. Indeed, we measured a detectable increase of protein adsorption, from 1.25 to 1.55 mg/m^2, during the first 30 min after emulsion preparation (the accuracy is about ± 0.1 mg/m^2). No such change in the protein adsorption was detected for emulsions prepared with 0.1 wt % BLG, which explains why no significant initial increase of emulsion stability was found in this system.

Remarkably, we did not detect any tendency for increase or decrease of Γ at storage times longer than 30 min for both systems, 0.02 and 0.1 wt. % BLG. The measured drop size distributions and mean volume-surface diameters were virtually the same for all samples. Therefore, the decreased emulsion stability observed at storage times longer than several hours could not be explained by changes in the amount of adsorbed protein or by changes in the drop size. These results mean that the observed aging effect (decreased emulsion stability upon shelf-storage) is due primarily to <u>changes in the structure of the protein adsorption layer</u>, which make it less efficient as emulsion stabilizer. One possible explanation of the aging effect is that the formation of strong intermolecular bonds between the adsorbed protein molecules (see Figure 3) transforms the adsorption layer into a fragile shell, which ruptures upon surface extension. As explained in Ref. (1) the formation or the expansion in area of an emulsion film in the point of contact between two drops is accompanied with an extension of the drop surfaces. If the adsorption layer is fragile, then bare (deprived of protein) oil-water spots could appear on the film surfaces, leading to film destabilization and drop coalescence.
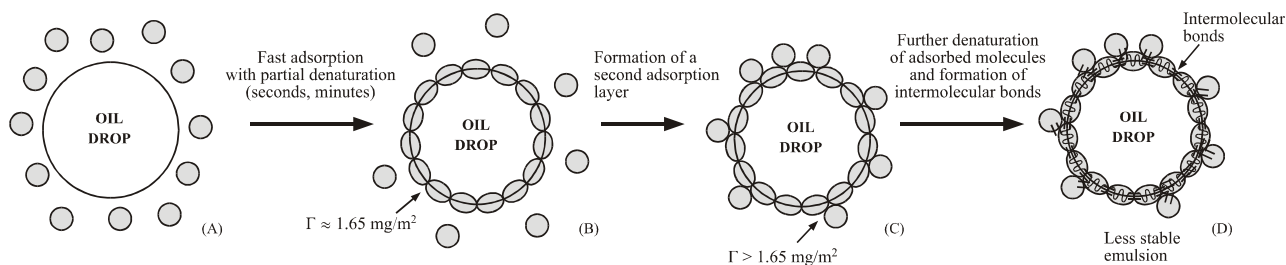

Figure 3. Schematic presentation of the processes which probably take place in the adsorption layer upon emulsion shelf-storage.

To check the effect of the intermolecular bonds in the adsorption layer on the aging effect, we performed a series of experiments on emulsion stability in the presence of various additives. The role of the <u>non-covalent interactions</u> was tested by addition of 4 M urea, which is known to break the H-bonds and to suppress the formation of hydrophobic bonds between the protein molecules. The obtained results for emulsion stability upon self-storage at three different concentrations of urea are presented in Table 1. As one can see, the short-term emulsion stability increases with the raise of the urea concentration. Furthermore, the addition of 4M urea is sufficient to prevent completely the aging effect (the long-term loss of stability) observed with BLG in the absence of urea.

Table 1. Critical osmotic pressure, P_{OSM}^{CR}, for BLG-containing emulsions in the presence of Urea.

Storage time	0.1 wt. % BLG			
	No Urea	3 M Urea	4M Urea	6 M Urea
30 min	9700 ± 1300	20 000	26 700 ± 1500	25 000 ± 1500
24 h	2500 ± 500	16 000	25 700 ± 1500	25 000 ± 1500
6 days	2000 ± 300	Not measured	25 700 ± 1500	25 000 ± 1500

As shown by Pace and Tanford (5), the addition of 4.4 M urea in the aqueous solution of BLG has little effect on the protein molecules in the bulk solution at room temperature, 22 °C - no significant denaturation is induced by urea of this concentration. This means that in our experiments, in which 3 or 4 M of urea are added to the protein solutions, the urea acts exclusively on the protein molecules incorporated in the adsorption layer. Even at 3 M urea, which is certainly insufficient to cause a detectable protein denaturation in the bulk solution (5), the aging effect was strongly suppressed.

The hypothesis that the aging effect is caused by the formation of strong non-covalent intermolecular bonds in the adsorption layer, is supported by the data of Fang and Dalgleish (6). By studying BLG emulsions with FT-IR spectroscopy, these authors found that the adsorbed protein molecules undergo significant changes during a 3-day period of shelf-storage: (1) the fraction of disordered regions in the adsorbed protein molecules becomes larger with time, and (2) intermolecular β-sheets were found to form and increase with time in the adsorption layer. Therefore, the formation of the fragile shell and the related reduction of stability in BLG-containing emulsions are most probably due to the slow development of these strong, non-covalent intermolecular bonds, which are suppressed by addition of urea.

To check the role of the covalent intermolecular <u>disulfide bonds</u> on the long-term stability of BLG emulsions, we performed the same type of experiments in the presence of 10 mM dithiothreitol (DTT) – a reagent known to block the formation of S-S bonds. We found that the addition of DTT to the protein solution had no effect on emulsion stability - the results were the same as those in absence of DTT. This result shows that the aging effect is not related to formation of disulfide bonds in the adsorption layer.

The fact that the BLG emulsions exhibit a significant aging effect (loss of stability with time), whereas the WPC emulsions preserve their stability practically unchanged for a long period of time, could be explained by the different procedures for production of these protein samples. The used WPC (AMP8000) is obtained by spray-drying, which certainly led to a partial denaturation of the protein molecules. Our study (2) showed that the protein molecules in WPC do not form extensive intermolecular bonds in the adsorption layer. In this aspect, the BLG molecules are very different – they change significantly upon adsorption and strong intermolecular bonds develop in the adsorption layer with time. More detailed explanations are given elsewhere (2).

3.3. Effect of thermal treatment of the emulsions on their stability. The thermal treatment is another factor, which is known to lead to conformational molecular changes and formation of intermolecular bonds in the adsorption layer (6-8).

To check how the thermal treatment affects the long-term stability of BLG-containing emulsions,

a series of experiments with heated emulsions was performed. These emulsions were prepared by the following protocol: (1) The temperature of freshly prepared emulsions was raised from room temperature up to 78 °C for 10 min by using a thermostat; (2) The emulsions were stored at 78 °C for 5 min; (3) The emulsions were taken out of the thermostat and stored at room temperature for different periods of time, varying from 2 hours to 6 days; (4) At the end of the storage period, the emulsions were centrifuged for characterization of their coalescence stability. In parallel, the protein concentration in the serum was determined in other emulsions, prepared under equivalent conditions.

The obtained results for P_{OSM}^{CR} and Γ of heated emulsions, stabilized by 0.1 wt. % BLG are presented in Table 2. As one can see, the protein adsorption decreases after emulsion heating to the value, which corresponds to a dense adsorption monolayer. This is probably due to desorption of molecules from the second adsorption layer during heating. However, as one can see from Table 2, the emulsion stability is significantly higher for heated samples as compared to that for the non-heated ones. No effect of aging (loss of stability upon storage) is observed for the heated samples - the emulsion stability remains virtually constant for storage period between 2 h and 6 days.

The observed increase of emulsion stability upon heating can be explained with the formation of an elastic network of adsorbed protein molecules, covalently bound by disulfide bonds (7-9). Indeed, one can expect that the formation of a network of disulfide bonds creates rather different structure and properties of the adsorption layer as a result of heating (9). Such an elastic adsorption layer of covalently bound molecules is expected to provide much more efficient protection of the drops against coalescence, because no rupture of the layer is expected to occur upon small or moderate surface expansion.

In other words, the obtained results indicate that the aging effect is related mainly to formation of non-covalent bonds, which make the adsorption layer fragile (i.e. the emulsions become less stable), whereas the heating probably leads to development of covalent disulfide bonds, which create an elastic network on the surface (more stable emulsion).

Table 2. Critical osmotic pressure, P_{OSM}^{CR}, and protein adsorption at the oil drop surface, Γ, for non-heated emulsions (non-shaded columns) and heated emulsions (shaded columns) at 0.1 wt % BLG.

Aging time, min	P_{OSM}^{CR}, Pa		Γ, mg/m^2	
	Non-heated	Heated	Non-heated	Heated
2 h	9700 ± 1300	40 000 ± 3000	2.9 ± 0.8	1.7 ± 0.5
24 h	2500 ± 500	40 000 ± 3000	2.9 ± 0.8	2.2 ± 0.5
6 days	2000 ± 300	40 000 ± 3000	2.9 ± 0.8	2.0 ± 0.5

Noticeably, the heating of WPC emulsions by the described protocol did not change their stability.

4/ Conclusions.

♦ The emulsion stability increases in a step-wise manner with protein adsorption for WPC-containing emulsions. The threshold value of surface coverage, required for obtaining stable emulsions is θ* ≈ 0.8, which is close to the value obtained with BLG emulsions, θ* ≈ 0.9 (1).

- The stability of BLG-containing emulsions significantly decreases after one day of shelf-storage, as compared to the stability of freshly prepared emulsions. This phenomenon is termed "the aging effect" and is not related to changes in the mean drop size or the protein adsorption on drop surface.

- The obtained results suggest that the aging effect is caused by the development of strong lateral non-covalent bonds (H-bonds and hydrophobic interactions) between the adsorbed protein molecules, which leads to the formation of a fragile protein shell on drop surface.

- The thermal treatment of BLG emulsions increases the short-term emulsion stability, while the aging effect disappears. The increased stability upon heating could be explained with the formation of an elastic network of denatured BLG molecules, which are cross-linked by disulfide bonds.

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