Microstructure of Whey Protein/ Anhydrous Milkfat Emulsions

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Abstract

Research has been undertaken in order to determine the microstructure of emulsions consisting of whey proteins and anhydrous milk fat. The results revealed that whey protein films were formed at oil/water interfaces. Scanning electron microscopy (SEM) analysis of chemically fixed specimens, and cryo-SEM analysis of partially etched, frozen specimens revealed that the adsorbed protein layer (coating the fat droplets) had a granular, aggregated appearance. Emulsions with a high fat load prepared under high homogenization pressure exhibited a bimodal particle-size distribution. Clustering of the protein-coated droplets, and sharing phenomenon at the oil/protein interfaces were evident in these emulsions. The structural analysis indicated that the bimodal distribution could be attributed to clustering of protein-coated fat droplets rather than to coalescence.

Key Words: Whey proteins, anhydrous milkfat, emulsion, microstructure, homogenization, clustering, sharing phenomenon, scanning electron microscopy (SEM), cryo-SEM, transmission electron microscopy.

Introduction

Whey proteins are widely used as functional ingredients in food systems [5]. The physicochemical properties of these proteins in general, and their emulsification properties in particular, have been extensively studied [2, 4, 5, 7, 10, 13, 16-19, 21, 24, 27-29]. However, most of the reported studies used vegetable oils or model compounds as the dispersed phase at concentrations lower than those customarily used in dairy and other food emulsions.

The formation of a stabilizing protein film at oil/water interfaces has been studied and reported [9, 13, 14, 17]. These studies demonstrated that the stability and properties of whey protein-stabilized emulsions are affected by the composition of the emulsion and by homogenization parameters.

Emulsification of anhydrous milkfat by whey proteins may provide food processors with possibilities in different applications such as recombination of milkfat globules [23], preparation of various composite whey proteins/anhydrous milkfat gels [26], etc. One novel application for whey proteins, that is currently being studied, is their use as microencapsulating agents. The key to a successful microencapsulation process is the formation of a stable emulsion in which the encapsulated material (the core) is dispersed in a solution of the encapsulating (wall) agent [25]. The formation of whey protein stabilizing films at the core/wall interfaces is thus important.

There is a lack of information regarding the microstructural features of whey proteins/anhydrous milkfat based emulsions. Research has been undertaken in order to determine the microstructural features of emulsions consisting of high concentrations of anhydrous milkfat emulsified in whey protein solution, and to obtain information regarding the effects of homogenization conditions on the properties of the emulsions.

Materials and Methods

Whey protein isolate (WPI) and anhydrous milkfat (AMF) containing 99.8% fat (supplier data) were purchased from Le Sueur Isolates (Le Sueur, MN) and California Cooperative Creamery (Hughson, CA), respectively.
Preparation of emulsions

A solution of 5% (weight/weight, w/w) WPI in deionized water was prepared at 25°C. AMF was emulsified in the protein solutions at 30% and 50% (w/w). The emulsification was carried out in two stages. A coarse emulsion was prepared using an Ultra-Turrax T-25 (IKA Works, Cincinnati, OH) homogenizer operated at 13,500 rpm for 30 seconds. The second stage consisted of four to twelve successive homogenization steps using a Mini-Lab type 8.30H high pressure homogenizer (APV Rannie, St. Paul, MN) operated at either 50 or 80 MPa. The emulsion constituents were heated to 50°C prior to the emulsification and this temperature was maintained throughout the emulsification process.

The mean particle size of the emulsions was determined using a Malvern Mastersizer MS20 (Malvern Instruments, England). A 2 mW He-Ne laser beam (633 nm) and a 45 mm focus lens were used. In all cases, sizing of the emulsions was conducted using the 0807 presentation code of the instrument. Particle size distribution is presented in this study by volume frequency distribution of the AMF droplet diameter.

Scanning electron microscopy (SEM)

Emulsion specimens were prepared in agar "tubes" in a way similar to that described by Allan-Wojtas and Kalab [2]. An emulsion sample was aspirated into a glass Pasteur pipet. The lower end of the pipet was sealed with a drop of 3% agar sol at 40°C. After the agar was solidified, the pipet was dipped and rotated in 3% agar sol at 40°C to form a uniform thin layer of agar on the glass surface. The agar tube was then trimmed at its upper end and the pipet was withdrawn, leaving the sample in the agar tube. The open end of the gel tube was then sealed with drops of agar sol. The specimens were fixed in 4% glutaraldehyde in 0.1 M imidazole-phosphate buffer at pH 7 and 4°C for 12 hours. The fixed specimens were postfixed with 2% osmium tetroxide in the same buffer for 4 hours at 25°C. The fixed specimens were washed three times with the same buffer and then dehydrated using a series of 50%, 70%, 80%, 90%, and 100% (volume/volume, v/v) ethanol solutions. Dehydration was carried out for 30 minutes in each of these solutions. Preliminary study indicated that no difficulties were associated with the above dehydration procedure in comparison to a procedure that included 30% ethanol solution as the first stage. The specimens were frozen in Freon 22, freeze-fractured under liquid nitrogen, and then dried by critical-point drying using carbon dioxide as carrier.

The dry specimens were mounted onto specimen stubs using colloidal silver paint, gold-coated (5 nm), and analyzed microscopically using an ISI-DS 130 scanning electron microscope (Topcon Technologies, Pleasanton, CA) operated at an accelerating voltage of 15 kV.

Cryo-scanning electron microscopy (cryo-SEM):

A Hitachi S-800 (Tokyo, Japan) SEM equipped with a SP 2000 cryo-unit (EMScope, London, U.K.) was used. Emulsion samples were mounted on the cryo-specimen holder, cryo-fixed in liquid nitrogen, transferred to the cryo-unit in the frozen state, fractured using the in-situ fracturing knife, sublimed at -80°C for 3 minutes, cooled to -196°C, coated with platinum using the cryo-unit sputter coater, transferred to the microscope cold stage, and examined at 2 kV.

Transmission electron microscopy (TEM)

Emulsion samples were fixed, postfixed and dried in ethanol as described for the SEM analysis. After dehydration in ethanol, the specimens were embedded in Medcast quick-mix epoxy (Ted Pella, Redding, CA). Thin sections (90 nm) of the specimens were stained with uranyl acetate and lead citrate (Ted Pella, Redding, CA). The specimens were examined using a model EM109 Zeiss TEM (Carl Zeiss, New York, NY) operated at 80 kV.

Results and Discussion

Specimen preparation techniques can induce structural changes that either obstruct structural details or alter the original structural features of the emulsion [15]. In order to avoid misinterpretation of structural details, and in order to identify specimen-preparation-related artifacts, three independent electron microscopy techniques along with three different specimen preparation techniques were used.

Figure 1 is a representative cryo-scanning electron

Figure 1. Cryo-scanning electron micrograph of WPI/AMF emulsion. Emulsion composition: 5% (w/w) WPI and 30% (w/w) AMF. Homogenization conditions: Four passes at 50 MPa. Films of whey proteins (PM), solidified anhydrous milk fat (SF), unfractured, protein-coated fat droplets (UFD), and empty whey protein film (EPM) are visible.

Figure 2. Particle size distributions of WPI/AMF emulsions of different fat loads prepared under different homogenization conditions. In all cases, WPI concentration was 5% (w/w).

Figure 3. Scanning electron micrograph revealing the structure of a WPI/AMF emulsion. Emulsion composition: 5% (w/w) WPI and 30% (w/w) AMF. Homogenization conditions: Four passes at 50 MPa. PM: protein film; CS: cross-section through WPI-coated AMF droplet from which fat has been removed; SFD: shrunk, protein-coated AMF droplets; BP: bulk whey protein matrix.

Figure 4. Scanning electron micrograph revealing the structure of WPI-coated AMF droplets. Emulsion composition and homogenization conditions were as described in Fig 3. WCF: whey protein coated AMF droplet; D: damaged protein film; PM: protein film; BP: bulk whey proteins; CS: a cross-section through a protein-coated fat droplet from which fat has been removed. 1 and 2 are reference numbers given to each droplet (see text).

Figure 5. Scanning electron micrograph of a WPI/AMF emulsion sample revealing partially damaged WPI membrane. Emulsion composition and homogenization conditions were as described in Fig 3. WPM: whey protein film; CS: outer surface of the protein film; IS: inner surface of the protein membrane; V: void that represents where milkfat was located.
Emulsification by whey proteins

Micrograph of an AMF/WPI emulsion. Films of whey proteins (PM) around the solidified (frozen) AMF are clearly visible. The micrograph reveals both intact and fractured milkfat droplets coated with a WPI layer. In some cases the solid fat droplets fell out of the protein film as a result of the fracturing and empty protein envelopes (EPM) can be seen. The outer surfaces of the WPI films have a rough and granular appearance. These features probably represent protein aggregation at the interfaces. Similar granular protein layers at fat/water interfaces in dairy systems were reported by Ootwijn et al. [23] and Mulder et al. [20]. Liboff et al. [15] reported that the shape of milkfat droplets was distorted by specimen preparation technique. Our micrographs reveal the spherical shape of the AMF droplets, indicating that they were probably not greatly affected by the preparation procedure. Fig. 1 reveals that in some cases the protein layer was partially detached from the AMF droplet. This can be probably attributed to effects of the freezing or the fracturing steps associated with the preparation procedure.
Emulsification by whey proteins

Figure 6. Transmission electron micrograph revealing the structure of a WPI/AMF emulsion. AP: aggregated WPI adsorbed at the interfaces, FD: anhydrous milk fat droplet; USF: unsaturated AMF fraction; SF: saturated milk fat fraction. 1 and 2 are numbers of fat droplets (see text). Emulsion composition and homogenization conditions were as described in Fig. 3.

Figure 7. Clustering in WPI/AMF emulsions as revealed by SEM. Emulsion composition: 7a) 5% (w/w) WPI, 50% (w/w) AMF; 7b) 5% (w/w) WPI, 30% (w/w) AMF. Homogenization conditions: 7a) twenty passes at 80 MPa, 7b) 50 MPa. C: clustered protein-coated AMF droplets; PM: protein films; S: sharing; FD: fractured WPI-coated AMF droplets (fat removed).

Figure 8. Structural details of a "clustered" emulsion. Scanning electron micrograph of the emulsion presented in Fig. 7a under high magnification. C: cluster; PM: protein film; S: sharing of an interfacial protein film.

Figure 9. Transmission electron micrographs of a "clustered" emulsion revealing sharing of protein layers at the interfaces of fat droplets (Fig. 9a), and the structural details of the protein film shared by two fat droplets (Fig. 9b). USF: unsaturated fraction of AMF; SF: saturated fraction of AMF; PM: protein films; FD: fat droplet; S: sharing phenomenon. The aggregated nature of the WPI layer at the interface is evident.

The particle-size distribution of the emulsion as analyzed immediately after homogenization (four passes at 50 MPa) exhibited a normal distribution of AMF droplets with a mean diameter of 0.6 μm (Fig. 2). The dimensions of the droplets as observed microscopically (Fig. 1) are in agreement with those determined using the laser beam device. Figures 3-5 show the structure of a chemically fixed AMF/WPI emulsion consisting of 5% WPI and 30% AMF homogenized at 50 MPa (four successive passes through the homogenizer). Fig. 3 reveals the structural features of a typical emulsion sample observed at relatively low magnification. Protein films around the milkfat droplets can be seen. However, the micrograph reveals that although some intact fat droplets can be observed, a significant amount of the AMF has been removed from the fractured droplets during the various stages of specimen preparation. In these cases, fragments (usually hemispheres) of the protein films can be seen. In other cases, relatively well-preserved interfacial protein films can be observed. Some of these films exhibit holes (probably caused by the fracturing) through which the fat may have been removed during the critical-point drying. Varying degrees of shape distortion and shrinkage of the coated droplets can be seen in Fig. 3. These artifacts may be attributed to the specimen preparation procedure.

Intact and fractured WPI-coated, AMF droplets are visible in Fig. 4. The protein films at the interface exhibit a granular and aggregated nature similar to what was observed with cryo-SEM. The intact AMF droplets, as well as those less damaged by the fracturing step, reveal that in many cases (observed in many micrographs) the spherical shape of the fat droplet was well-preserved during the preparation procedure.

The SEM analysis of chemically fixed, fractured, emulsion specimens involves certain difficulties that may lead to errors. In many cases, only a cross-section through the coated fat droplet can be observed. The shape of the cross-section does not necessarily represent the shape or size of the entire droplet, but rather the shape of the droplet part that has been removed by fracturing. Only in cases where a relatively large part of the droplet is visible, can the features of the droplets and the position of the fracture plane be determined. Structural features of chemically fixed and fractured emulsion samples should thus be carefully analyzed while considering the effects of different parameters, including the position of the AMF droplets relative to the fracturing plane and the mechanical properties of the fixed specimen. Droplets 1 and 2 in Fig. 4 provide examples of different shapes of "openings" in the protein layer that coated the original fat droplets. In these cases, relatively small parts of the protein films have been removed during fracturing and large parts of the protein-coated fat droplets are visible. This allows identification of the position of the fracture plane relative to the entire droplet, and hence conclusions regarding the shape and size of the droplets are possible. It should be mentioned that using stereo pairs can greatly help to determine the position of the fracture plane and the relative heights of the structural features. The presented micrographs indicate that even in cases where the fat droplets have been either removed or partially shrunk as a result of the preparation procedure, the original dimensions and shape of the fat droplet can be estimated based on the dimensions and shape of the crater in which the droplet was embedded. The crater can be considered as a "footprint" representing the dimensional features of the AMF droplet as preserved by the fixation procedure (Figs. 3 and 4).

Figure 5 shows a large part of a whey protein-coated AMF droplet from which the fat has been removed. This micrograph reveals the existence of a continuous interfacial protein film that is 50-100 nm thick and granular in nature. The granular nature of the protein layer observed in Fig. 5 corresponds with the structural features revealed (at a lower magnification) by the cryo-SEM investigation. Although significant fat removal was associated with the specimen preparation for SEM, the protein films at the oil/water interfaces seem to be well-preserved.

Figure 6 is a transmission electron micrograph revealing the structure of the AMF/WPI emulsion. Although the spherical shape of the AMF droplets was well-preserved in most cases, a few fat droplets exhibit some angular shape distortions similar to those reported by Liboff et al. [15]. Shape distortion can be attributed to the effect of the preparation technique as well as to the effects of fat crystals on the protein film [15]. Continuous, well-defined protein membranes can be seen around the fat droplets, and the aggregated nature of the protein film adsorbed to the fat droplets is evident.

The transmission electron micrograph reveals differences in staining intensity within the fat droplets. This phenomenon is attributed to the heterogeneous composition of milkfat [26]. The unsaturated fraction of the fat is
known to interact well with osmium tetroxide and hence to become heavily stained, while the saturated fraction is poorly stained by the osmium reagent [11, 15, 26]. The saturated, lightly stained fat fraction can usually be found in the central part of the fat droplets surrounded by the unsaturated, well-stained fat fraction, forming a layered type of structure. Although such a distribution is demonstrated by many of the fat droplets in Fig. 6, some cases in which almost the entire cross-section of a fat droplet is well-stained are observed (fat droplets 1 and 2). These observations can be linked to the position of the sectioning plane relative to different droplets in the sample. The layered structure may represent cases where the cutting plane was located relatively far from the peripheral area of the fat droplet so the presence of the two fractions is evident. The less frequent cases, in which the lightly-stained core is not observed, represent situations in which the cutting was through the peripheral layer composed of the unsaturated fat. Although similar observations were reported by Ruth and Kinsella [26] and other, it has been suggested that this layered arrangement represents an artefact associated with the fixation process [31]. Liboff et al. [15] demonstrated that it was possible to observe the high-melting glyceride crystals evenly distributed throughout the fat globule, but also showed that the "native" structure can be lost as a result of the fixation procedure. The layered structure observed in the present study can thus be considered as an artefact of the fixation stage rather than the actual distribution of the different lipid fractions of the AMF.

Different buffered osmium tetroxide reagents have been suggested for staining emulsified fat. Among these are phosphate buffer [2], phosphate/imidazole buffer [3, 15], and veronal-acetate/imidazole buffers [1, 12]. Based on these cited studies, it has been demonstrated that unsaturated fatty acids react well with imidazole buffered osmium tetroxide. Our results confirm that observation.

**Clustering and sharing phenomena**

Emulsions homogenized under high pressure and/or by 12 successive passes through the homogenizer were characterized by a bimodal particle distribution rather than a normal (unimodal) distribution (Fig. 2). Two different mechanisms, coalescence or aggregation are known to be associated with the effects of homogenization on dairy emulsions and could have led to the bimodal distribution [6, 9]. Coalescence results in the formation of fat droplets larger than those obtained under milder homogenization conditions, while aggregation (or clustering) is associated with bridging of two or more protein-coated fat droplets. Such aggregates may show sharing phenomena at the interfaces of the dispersed droplets.

Sharing phenomenon in emulsions is a situation in which two or more fat droplets share parts of the same stabilizing film at their interfaces. A shareable, surface-active material is one that can be adsorbed to one fat droplet in a way that leaves part of the molecule free to be adsorbed onto other interfaces [22]. According to the clustering-through-sharing theory, fat droplets that are only partially coated by a shareable protein film when leaving the homogenizer pressure valve can interact with each other to form clusters. It has been suggested that sharing and the resulting clustering is favored by limiting concentrations of the surface-active material during the homogenization [22, 30]. Clustering may result from a dramatic increase in interfacial surface area and/or a low concentration of the surface-active material available for adsorption at the newly-formed interfaces.

The microstructure of emulsions containing 50% and 30% AMF, homogenized at 80 and 50 MPa (twelve successive passes) is presented in Figs. 7a and 7b, respectively. A higher magnification micrograph of the emulsion presented in Fig. 7a is provided in Fig. 8. As stated above, the milkfat has been removed during the specimen preparation process, but parts of the protein films below the fracture plane have been preserved and can be analyzed.

Analysis of micrographs such as Figs. 7 and 8 revealed no evidence that coalescence occurred during homogenization. The "C" areas in these micrographs reveal clusters of fat droplets in which sharing phenomena are evident. Protein films associated with two fat droplets are clearly observed. Since each side of the protein film is adsorbed to a different fat droplet, the phenomenon is called "sharing". The structural features of the emulsions thus provide an indication that clustering (or aggregation) rather than coalescence is associated with the bimodal fat particle size distribution. These observations are in agreement with the theory of clustering through sharing in emulsions [8, 22, 30].

Whey proteins are characterized by an even distribution of hydrophobic and hydrophilic domains [13]. Such a distribution of hydrophobic segments may favor sharing phenomena in oil/water type emulsions. At early stages of the adsorption of whey proteins onto fat droplet surfaces and before completion of the unfolding and spreading associated with adsorption [13], it can be assumed that there are hydrophobic areas that remain unengaged in hydrophobic interactions. These segments of the protein molecules are thus free to interact with an approaching, partially coated fat droplet to form a cluster in which interfaces of the two droplets share the same stabilizing film. This may also suggest that in the case of whey proteins, reaching a limiting concentration of the stabilizing protein is less critical to the induction of clustering than in the case of other proteins characterized by uneven distribution of their hydrophobic segments. Relatively high homogenization pressure and/or successive passes through the homogenizer at relatively high fat loads (30 and 50% in the present study) favor conditions in which sharing can be manifested as a result of the enormous increase in interfacial surface area. The results indicate that clustering can be induced even at 50 MPa and 30% fat load after 12 homogenization steps, however, no attempt has been made to quantify the extent of clustering through sharing as a function of the homogenization conditions. Clusters which do not show sharing phenomena are also present in Fig. 7a and 7b. These cases may represent the results of protein-protein interactions between fully coated AMF droplets. No attempt has been made to investigate the extent of the formation of such clusters as a function of the homogenization conditions. In a separate study [32], differences in the flow curves of the emulsions presented in Figs. 7a and 7b were found. An
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increase in viscosity was observed in all cases where bi-modal particle size distribution and clustering were identi-fied. Similar observations have been reported by Walstra [31] regarding clustering of milkfat globules during cream homogenization. In addition, emulsions of different fat to protein ratio that were homogenized under identical condi-tions (that have been found to promote clustering) exhibited different particle size distributions as well as different flow curves (32). These differences may indicate different extent of clustering associated with these emulsions.

Figures 9a and 9b are transmission electron micrographs of a specimen prepared from the emulsion shown in Fig. 7a. The existence of a protein layer shared by two AMF droplets is clear and confirms the SEM results in this regard.

It should be noticed that the thickness of the protein layer around the AMF droplets varies considerably. In some cases, such as the one presented in Fig. 9, this layer is relatively thin (50 nm), while in other cases, e.g., in the clustered area highlighted in Fig. 8, a 150-200 nm layer is observed around some of the droplets. These differences can be related to clustering at different stages of the twelve homogenization steps. It has been reported that whey pro-teins form stable multi-layer structures at oil/water inter-faces [14]. The thicker protein layers probably represent cases in which the clustering and sharing occurred at a relatively late stage of the process and after the build-up of a relatively thick multi-layer protein film around one of the AMF droplets. Cases in which thin protein layer is observed may thus be attributed to clustering at an earlier stage at which less developed protein film was present at the interface.

Based on the results presented in Fig. 2, and 7-9, it appears that the clusters were not disrupted into their constituent fat droplets during particle size determination using the Mastersizer. Each cluster was depicted by the laser beam as one particle having the overall diameter of several aggregated fat droplets. This resulted in obtaining a bi-modal particle size distribution.

Conclusions

This study provides evidence that in emulsions consisting of anhydrous milkfat and whey proteins, a layer of aggregated whey proteins is formed at oil/water interfaces. The presence of this continuous, well-defined protein layer around the fat droplets is an indication of the good emulsi-fi-cation properties of whey proteins. Similar structural features of the whey protein films were revealed by SEM analysis of chemically fixed specimens and cryo-SEM analysis of partially sublimed, frozen specimens. The results indicate that although the protein films were well-preserved in the chemically fixed specimens, a significant proportion of the fat was removed during specimen preparation. In some cases, this was associated with artifacts related to the shape of the droplets.

Clustering of WPI-coated AMF droplets was observed when emulsions were prepared under conditions known to favor clustering. The microstructural details indicated that sharing phenomena were involved in cluster formation.

This study provides structural information related to the role of whey proteins in the emulsification of anhydrous milkfat. Such information could prove useful when whey proteins are being used in the reconstitution of milkfat globules or when they are considered as micro-encapsulating agents.

Acknowledgements

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References

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Discussion with Reviewers

B.L. Armbruster: I realize that low acceleration voltages are necessary to examine cryo-SEM samples, but 2 kV and 10,000X magnification are too low to resolve the structure of the whey protein coating.
Authors: As mentioned by the reviewer, using high acceleration voltage in cryo-SEM is problematic. Because of such difficulties and due to the delicate nature of the emulsions, we decided to use this technique in the present study only in order to confirm the results of SEM analysis of chemically fixed samples. The presented results indicate that similar structural features were revealed by SEM and cryo-SEM. This means that the structural details were relatively well-preserved by chemical fixation and no significant artifacts were introduced by the specimen preparation procedure.

B.L. Armbruster: Do you know the ratio of saturated to unsaturated fat present in the anhydrous milkfat? With this ratio you could use image analysis techniques to quantify stained versus unstained regions to determine if staining is really useful in differentiating between fats.
Authors: The authors agree with the suggestions of the reviewer, however, this was not among the objectives of the present study. We did not analyze the composition of the AMF. The discussion about the staining effects was provided in order to suggest explanation to our observations. Our observations and explanation are supported by observations of others (see references in text) and highlights the dispute in the literature regarding this issue.

L.J. Kiely: Please comment on the selective preservation of some fat droplets while others are removed.
Authors: The preservation of fat droplets seems to be a function of several parameters like the damage caused to the droplet during the fracturing stage, the mechanical strength of the individual protein layer at the interface, the thickness of the protein layer, and the position of the fracture plane relative to the fat droplet. The preserved structures represent those droplets that better survived the fracturing stage. In such cases, the fat was removed (during the critical-point drying stage) through a relatively small hole in the protein layer while the protein film was well preserved. In cases where no damage to the protein film has occurred during the fracturing, the entire droplet survived the preparation procedure (Fig. 4).

L.J. Kiely: What exactly does WPI consist of?
Authors: WPI consisted of 95.5% protein, 1.8% ash, and 2.7% moisture.