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Homogenization Conditions Affect the Oxidative Stability of Fish Oil Enriched Milk Emulsions: Oxidation Linked to Changes in Protein Composition at the Oil−Water Interface

ANN-DORIT M. SØRENSEN,[†] CAROLINE P. BARON,[†] METTE B. LET,[†] DAGMAR A. BRÜGGEMANN,[§] LISE R. L. PEDERSEN,[#] AND CHARLOTTE JACOBSEN*,[†]

Department of Seafood Research, Danish Institute for Fisheries Research, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark; LMC Centre for Advanced Food Imageing, Copenhagen University, DK-1958 Frederiksberg C., Denmark; and Department of Molecular Biology, Protein Chemistry Laboratory, University of Aarhus, DK-8000 Aarhus C, Denmark

Fish oil was incorporated into milk under different homogenization temperatures (50 and 72 °C) and pressures (5, 15, and 22.5 MPa). Subsequently, the oxidative stability of the milk and changes in the protein composition of the milk fat globule membrane (MFGM) were examined. Results showed that high pressure and high temperature (72 °C and 22.5 MPa) resulted in less lipid oxidation, whereas low pressure and low temperature (50 °C and 5 MPa) resulted in faster lipid oxidation. Analysis of protein oxidation indicated that especially casein was prone to oxidation. The level of free thiol groups was increased by high temperature (72 °C) and with increasing pressure. Furthermore, SDS-PAGE and confocal laser scanning microscopy (CLSM) indicated that high temperature resulted in an increase in β-lactoglobulin adsorbed at the oil−water interface. This was even more pronounced with higher pressure. Less casein seemed to be present at the oil−water interface with increasing pressure. Overall, the results indicated that a combination of more β-lactoglobulin and less casein at the oil−water interface gave the most stable emulsions with respect to lipid oxidation.

KEYWORDS: Homogenization; pressure; temperature; milk; protein oxidation; lipid oxidation; n-3 PUFA; milk fat globule membrane (MFGM)

INTRODUCTION

The marine n-3 polyunsaturated fatty acids (PUFA) have received increasing attention during the past 30 years. This is due to the findings that a high intake of marine PUFA has been observed to correlate with a lower risk of atherosclerosis, and subsequently a number of other beneficial health effects of n-3 PUFA have also been reported (*1*). In most Western countries the intake of n-3 PUFA is far too low, and it is a challenge to increase the intake of these healthy PUFA. One possible strategy is to substitute less unsaturated fats with n-3 PUFA-rich lipids such as fish oil in a range of food products. However, the polyunsaturated nature of fish oil makes them highly susceptible to lipid oxidation. Therefore, development of strategies to prevent lipid oxidation in fish oil enriched foods is necessary if such foods are to become successful in the marketplace (*2*). Fish oil enriched milk is one type of food in which the oxidative stability of the lipids and the development of strategies to reduce lipid oxidation have been extensively investigated (*3*−*6*). In a recent study it was observed that the oxidative stability of the lipids was influenced by the homogenization process. When high homogenization pressure and temperature were applied under incorporation of fish oil into milk, the oxidation of lipids decreased (*6*).

The initial step in lipid oxidation in emulsions takes place at the interface between the oil and water phases (*7*). The homogenization conditions affect the droplet size and thereby the total interfacial area in emulsions. Therefore, lipid oxidation might be expected to be faster in emulsions with small droplets, owing to the larger total interfacial area, compared to larger droplets. Some studies in emulsions support this hypothesis, as an increase in total interfacial area has been shown to accelerate lipid oxidation (*8, 9*); however, other studies have found the opposite (*6, 10*). In the study by Let et al. (*6*) a negative correlation between the total interfacial area and the degree of lipid oxidation was to some extent observed.

Milk is a complex liquid, which roughly can be considered to be composed of three phases: a water phase, a fat phase, and an interfacial layer. The milk fat is emulsified in the aqueous phase, and the other components in milk are either suspended (e.g., caseins) or dissolved (e.g., whey proteins) in the aqueous

* Corresponding author (telephone +45 4525 2559; e-mail cja@difres.dk).
[†] Danish Institute for Fisheries Research.
[§] Copenhagen University.
[#] University of Aarhus.

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Table 1. Fatty Acid Composition and Tocopherol Content of the Fish Oil Used and of the Prepared Milk Emulsions

fatty acid (%)	fish oil	milk emulsion
C14:0	3.1	7.3
C16:0	9.6	24.3
C18:0	2.1	8.8
Σ SAT	**15.4**	**40.4**
C16:1(n-7)	6.6	3.5
C18:1(n-7)	4.2	3.5
C18:1(n-9)	16.5	20.6
C20:1(n-9)	12.7	4.3
C22:1(n-11)	7.0	2.7
Σ MUFA	**49.1**	**36.2**
C18:2(n-6)	1.7	2.4
C18:3(n-3)	0.9	0.6
C18:4(n-3)	2.9	1.1
C20:4(n-3)	0.7	
C20:5(n-3)	9.5	3.6
C21:5(n-3)	0.5	
C22:5(n-3)	1.0	
C22:6(n-3)	12.0	4.4
Σ PUFA	**29.3**	**13.1**
other	6.2	10.3
α-tocopherol (mg/kg)	**334**	**0.8**

phase. The interfacial layer in milk is normally referred to as milk fat globule membrane (MFGM). The membrane is composed of proteins, phospholipids, glycolipids, cholesterol, glycerol, and water. In milk, this membrane functions as an emulsifier by covering the milk fat and stabilizing it against flocculation and creaming (*11*). During homogenization of raw milk the partitioning of the different components in the milk changes. Considerable amounts of protein from the aqueous phase, especially casein and β-lactoglobulin, adsorb to the interface, and the amount and type of adsorbed milk proteins can be affected by the conditions of homogenization, that is, pressure and temperature (*12*−*14*). In this study, we will refer to the oil−water interface after homogenization as MFGM despite these changes in the composition of the membrane. Because contradicting effects of the droplet size on lipid oxidation in emulsions have been observed, the composition and structure of the oil−water interface most likely play an important role with respect to oxidative deterioration in emulsions. Moreover, it has been shown that metal ions such as iron and copper also are important factors in the oxidative processes in emulsions, especially in PUFA-rich food emulsions (*15*, *16*). Changes in the protein composition at the oil−water interface due to homogenization might change the iron availability, for example, by affecting the chelation of iron at the interface (*7*, *17*−*19*). This aspect could be of considerable importance with respect to incorporation of fish oil into milk by homogenization and may thus affect the subsequent oxidative stability of the enriched milk emulsions.

Both lipids and proteins in fish oil enriched milk can be exposed to oxidative deterioration, but so far most studies have investigated only lipid oxidation. During oxidation of the lipids, the levels of peroxides and subsequently secondary volatile oxidation products increase, giving rise to an unpleasant fishy off-flavor (*3*, *5*). Protein oxidation involves formation of protein carbonyls, intra- and intermolecular cross bindings, and fragmentation (*20*, *21*) and might affect the nutritional value of the product by, for example, inducing loss in essential amino acids (*22*). The effect of the conditions of homogenization on protein oxidation has not been investigated yet but might have important consequences for the oxidative stability of the fish oil enriched food products.

Table 2. Experimental Design

homogenization	pressure (MPa)	temperature (°C)
milk + 0.5% fish oil	5	50
		72
	15	50
		72
	22.5	50
		72

A better understanding of how the homogenization process affects the oxidative stability of fish oil enriched milk will allow us to select the optimum conditions of homogenization for incorporation of fish oil in milk to obtain the most stable product. Therefore, the aim of this work was to evaluate the effect of the conditions of homogenization on the oxidative stability of both lipids and proteins in n-3-enriched milk (o/w emulsion). Six emulsions were produced at different pressures (5, 15, and 22.5 MPa) and temperatures (50 and 72 °C), and their oxidative stability was evaluated. In addition, changes in the protein composition of the MFGM were examined.

MATERIALS AND METHODS

Materials. Fresh milk (0.5 and 1.5% fat contents) was purchased in a local supermarket. Cod liver oil was supplied by Maritex A/S, Sortland, Norway. The fatty acid composition and tocopherol content of the fish oil and the prepared milk emulsions are given in **Table 1**.

Ellman's reagent [6,6′-dinitro-3,3′-dithiodibenzoic acid (DTNB)], 2,4-dinitrophenyl hydrazine (DNPH), 1,4-dithiothreitol (DTT), pararosanilin base, activated charcoal, and Nile Red were obtained from Sigma-Aldrich (Steinheim, Germany). Trichloroacetic acid (TCA) and trifluoroacetic acid anhydride (TFA) were obtained from Merck (Darmstadt, Germany). Polyvinylpyrrolidone was purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Furthermore, bovine serum albumin (BSA) was also purchased from Sigma-Aldrich.

The primary antibodies and the secondary antibodies for immunofluorescence analysis were obtained from the following suppliers: anti-bovine casein (International, Saco, ME), anti-bovine lactoferrin (Biodesign, Bethyl Laboratories, Inc., Montgomery, TX), donkey anti-goat conjugated with Alexa 647 (Molecular Probes Inc., Eugene, OR), and anti-sheep conjugated with fluorescein (Sigma-Aldrich, St. Louis, MO). The anti-DNPH antibody and the anti-rabbit Ig conjugated with horseradish peroxidase were both obtained from DakoCytomation Denmark A/S (Glostrup, Denmark). All solvents were of HPLC grade (Lab-Scan, Dublin, Ireland).

Emulsion Preparation. Milk with 1.5% fat and milk with 0.5% fat were mixed (1:1, v/v) to obtain a total fat content of 1%. Subsequently, the milk was pasteurized at 72 °C for 15 s, and the fish oil (0.5% v/v) was added. Thereafter, milk samples were homogenized with a two-valve Rannie homogenizer (Albertslund, Denmark) at different pressures and temperatures (**Table 2**). Milk samples homogenized at 72 °C were homogenized immediately after pasteurization, whereas milk samples homogenized at 50 °C were first put on ice and flushed with nitrogen to decrease the temperature to 50 °C prior to homogenization. Obtained milk emulsions were stored in 250 mL sterilized Pyrex bottles at 2 °C in darkness. Samples for peroxide values (PV), GC-MS, protein carbonyls, and free thiol groups were taken after 0, 8, and 14 days of storage, flushed with nitrogen, and stored at −40 °C until further analysis except milk emulsions for the isolation of MFGM proteins. Here, the samples were taken after 1 day of storage, and isolation of the proteins was performed on the same day.

Droplet Size Determination. The size of the fat droplets in milk emulsions was determined by laser diffraction with a Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, U.K.). A few droplets of the milk emulsion were suspended directly in recirculating water (2800

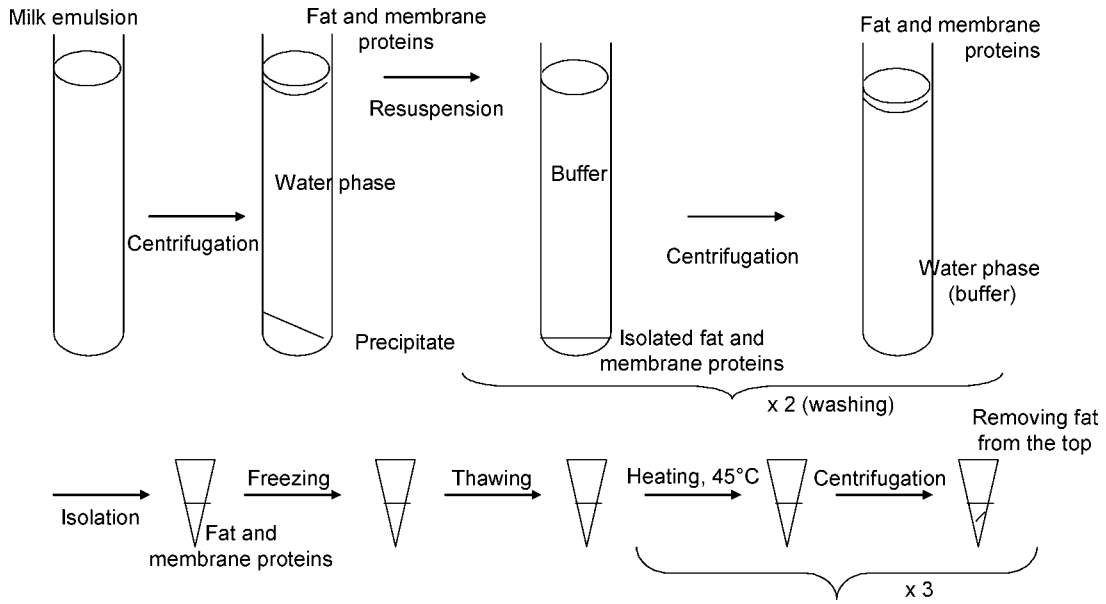

Figure 1. Schematic overview of the isolation of the proteins associated with the MFGM.

rpm, obscuration = 14−16%). The setup used was the Fraunhofer method, which assumes that all sizes of particles scatter with equal efficiencies and that the particles are opaque and transmit no light (*23*). The results were reported as surface mean diameter, $D[3,2] = \sum n_i d_i^3 / \sum n_i d_i^2$, where d is the diameter of individual droplets.

Confocal Laser Scanning Microscopy (CLSM). *Immobilization.* Immobilization of dairy protein and lipid droplets was achieved by dropping small pieces of poly(vinylidene fluoride) membrane (PVDF), pore size = 0.20 μm (Millipore Corp., Billerica, MA) into the milk emulsions. Subsequently, the membrane pieces were transferred to 4% paraformaldehyde in 50 mM phosphate-buffered saline (PBS), pH 6.7, for fixation.

Immunofluorescence. The membranes were blocked in 8% BSA in PBS. Thereafter the primary and secondary antibodies were successively applied to the membrane in 2% BSA in PBS. Lipid droplets were labeled using 0.001% Nile Red in PBS, pH 6.7. The samples were mounted in aqueous medium consisting of 10 M polyvinylpyrrolidone in Tris buffer (100 mM, pH 7.4) and were viewed using a confocal laser scanning microscope (TCS4d, Leica Laser Technik GmbH, Heidelberg, Germany) equipped with an argon/krypton laser and ×100 plan apo (1.4 NA) and ×40 plan apo (1.25 NA) objectives. The following filter settings were used: anti-sheep fluorescein, excitation wavelength = 488 nm, emission = 515−545 nm; Nile Red, excitation wavelength = 568 nm, emission = 580−720 nm; and Alexa fluor 647, excitation wavelength = 647 nm and emission >665 nm. To avoid crosstalk between the different dyes by simultaneous image acquisition, the individual parameter settings of the individual dyes were executed in a sequential order. During image acquisition each line was scanned eight times and averaged. The obtained images are the results of projections from stacks of images in vertical direction.

Differences in the Composition of MFGM Proteins. *Isolation of MFGM Proteins.* One day after the production of the fish oil enriched milk, the fat was separated from the milk emulsions by ultracentrifugation at 70000*g* and 15 °C for 1.5 h (L8-70M Ultracentrifuge, Beckman, Fullerton, CA). The water phase and precipitate were stored separately at −18 °C until further analysis. The fat phase was washed twice with 35 mL of buffer (10 mM imidazole, 10 mM acetate, pH 6.7) and frozen at −18 °C. Subsequently, the fat phase was thawed, heated to 45 °C (5 min), and centrifuged for 3 min at 13000*g* (Biofuge Pico, Heraeus, Hanau, Germany) to separate the lipids and MFGM proteins. The isolated MFGM proteins were dissolved in 4 M urea before storage at −18 °C until further analysis. A schematic overview of the isolation of the MFGM proteins is given in **Figure 1**.

Protein Determination. The MFGM fraction, water phase, and precipitate were dissolved in 2 M urea, and the protein concentration was determined spectrophotometrically (UV 160, Shimadazu, Columbia, MD) at 595 nm using Bradford reagent. The protein concentration was calculated using BSA as standard. The water phase, precipitate, and isolated MFGM proteins were diluted to a protein concentration of 1 mg/mL in 4 M urea.

MFGM Protein Separation by SDS-PAGE. The MFGM protein samples were diluted 1:1 with urea sample buffer [4 M urea, 0.05 M Tris, 2 M thiourea, 3% (w/v) SDS, 0.05% bromophenol blue, and 1% DTT]. Samples (20 μL) were loaded on a NuPage 10% Bis-Tris gel (Novex, Invitrogen, Carlsbad, CA). The gels were run in either MOPS or MES running buffer (Invitrogen) for 35 or 50 min at 200 V and subsequently stained with Coomassie Brilliant Blue R-250 (CBB) or periodic acid−Schiff (PAS). Quantity One 4.0 (Bio-Rad, Hercules, CA) was used for analysis of the gels.

Measurement of Peroxide Values. PV was determined as a measure of lipid oxidation. Lipids were extracted from the milk emulsions according to the methods described by Bligh and Dyer (*24*) using a reduced amount of solvent (*25*). The peroxide values were determined in the lipid extract by the colorimetric ferric−thiocyanate method according to the International IDF Standards (*26*).

Protein Oxidation. *Determination of Protein Carbonyls by UV Spectroscopy.* Protein carbonyl groups in the milk emulsions were determined using DNPH as previously described by Levine et al. (*27*). Briefly, proteins were precipitated 1:1 (v/v) with TCA and incubated in 10 mM DNPH in 2 M HCl for 30 min at room temperature. The pellet was washed in ethanol ethyl acetate (1:1, v/v) to remove unreacted DNPH and then redissolved in guanidine (6 M). Protein carbonyls were measured at 370 nm.

Measurement of Protein Carbonyls by Western Blot. Labeling of oxidized protein was conducted according to the method described by Levine et al. (*27*). Milk emulsions were diluted in Tris-HCl buffer (50 mM, pH 6.7) and 12% (w/v) SDS in the ratio 1:2:3 (v/v/v). To 60 μL of this mixture was added 60 μL of 20 mM DNPH dissolved in 10% (v/v) TFA. The reaction was conducted for 15 min in the dark at room temperature and stopped by the addition of 120 μL of neutralization solution [1.85 M Trizma base, 28% (v/v) glycerol, and 100 mM DTT]. DNPH-labeled samples were loaded on NuPage 10% Bis-Tris gel (Novex, Invitrogen). Subsequently, the proteins were transferred onto a PVDF membrane at 30 V for 1 h. The membrane was blocked with 5% (w/v) skimmed milk and incubated with the primary antibody at a 1:5000 dilution in 1% (w/v) skimmed milk for 1 h. Finally, the membrane was incubated with the secondary antibody at a 1:5000 dilution in 1% (w/v) skimmed milk. Oxidized proteins were transferred to autoradiograph paper using an ECL+ detection kit (Amersham Biosciences, Buckinghamshire, U.K.).

Table 3. Droplet Size (D[3,2], μm) of the Fat Droplet in the Different Milk Emulsions Measured by Laser Diffraction after 8 Days of Storage at 2 °C ($n = 2$)

milk emulsions		
temperature (°C)	pressure (MPa)	D[3,2]
50	5	1.61
	15	1.49
	22.5	1.34
72	5	1.09
	15	1.01
	22.5	0.53

Measurement of Free Thiol Groups. Free thiol groups were measured in the milk emulsions using Ellman's reagent according to the method described by Stapelfeldt et al. (*28*).

Statistical Analysis. To correlate the different analytical data, ANOVA partial least-squares regression (PLSR) was employed using Unscrambler 9.1 (Camo, Oslo, Norway). The design variables (X variables) were pressure (5, 15, and 22.5 MPa) and temperature (50 and 72 °C). The measured values used as Y variables were droplet size, PV, protein carbonyls, and free thiol groups. All variables were weighted [1/standard deviation (STD)], and the model was validated using cross-validation. The analysis was performed on a short thick data matrix (time varies along the Y variables), that is, one Y variable for each measured variable at each time point.

RESULTS

Droplet Sizes. The different conditions of homogenization significantly affected the size of the droplet in the n-3 PUFA enriched milk emulsions (**Table 3**). Diameters of the fat droplets varied from 0.53 to 1.61 μm, and both homogenization temperature and pressure influenced the size of the fat droplets. The largest droplet size was detected in the emulsion prepared at low temperature (50 °C) and pressure (5 MPa), whereas the smallest droplet size was obtained in the emulsion prepared at 72 °C and 22.5 MPa.

Changes in the Protein Composition of the Oil−Water Interface. *Evaluated by Confocal Laser Scanning Microscopy.* Antibody labeling toward milk proteins revealed that lipid−protein as well as protein−protein interaction in milk was strongly influenced by the homogenization conditions applied to the fish oil enriched milk. In **Figure 2**, colocalization of lipids and casein appears turquoise, whereas colocalization of casein and lactoferrin appears orange. As seen by the turquoise staining, the casein was readily seen on the surface of the lipid droplets in the milk emulsion homogenized at 50 °C and 5 MPa (**Figure 2a**). Larger green-stained casein aggregates were also visualized on the surface of the oil droplets. In these samples, no lactoferrin was observed. In contrast, samples that were homogenized at 72 °C and 22.5 MPa showed a substantial number of lactoferrin aggregates, which interacted with casein aggregates and small lipid droplets (**Figure 2b**). Interestingly, only a few larger droplets showed some restricted areas with casein at the oil−water interface, indicating that the presence of casein at the oil droplet surface was now much less pronounced. However, the number of casein aggregates was increased compared to the milk emulsion homogenized at low temperature and pressure.

Evaluated by SDS-PAGE. The protein composition in the isolated MFGM was determined by SDS-PAGE using two different staining methods: CBB and PAS. CBB staining is commonly used to stain proteins, but glycoproteins are stained only faintly or not at all with CBB. The MFGM contains glycoproteins, and to detect them and to reveal possible differences between treatments, PAS was used. The CBB-stained gels of isolated proteins from the MFGM are shown in **Figure 3**. The proteins were separated using MES running buffer and MOPS running buffer. The results showed that homogenization temperature influenced the amount of β-lactoglobulin and casein at the MFGM (**Figure 3**; **Table 4**). The high homogenization temperature (72 °C) resulted in a higher amount of β-lactoglobulin at the oil−water interface compared to the low homogenization temperature (50 °C), and the amount increased further with increasing pressure. Four different bands were observed for casein. Their molecular masses were estimated as 25, 28, 30, and 32 kDa, respectively. According to preliminary experiments (data not shown) and the literature, these proteins could be identified as κ-casein, β-casein, α_{s1}-casein, and α_{s2}-casein, respectively (*14*, *29*). Changes in the amount of the different caseins were small, but overall the amounts of caseins at the MFGM were lower when high pressure and temperature (72 °C and 22.5 MPa) were applied compared to the milk emulsion produced at lower pressures and temperatures (**Figure 3**; **Table 4**).

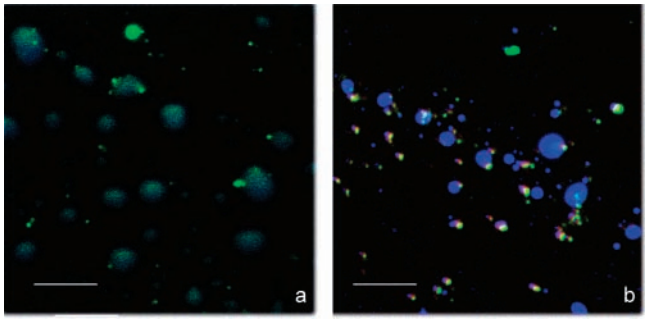

Figure 2. Antibody labeling of casein (green) and lactoferrin (red) of milk after different homogenization procedures showing different surface behaviors of the two proteins on lipid droplets (blue) in dependence of the homogenization treatment applied: (**a**) fish oil enriched milk homogenized at 50 °C and 5 MPa; (**b**) fish oil enriched milk homogenized at 72 °C and 22.5 MPa. Bar equals 7 μm.

No differences in the glycoprotein compositions of the MFGM were observed between the different emulsions after the gel had been stained with PAS (data not shown). Moreover, when the proteins from the water phase and precipitate were separated on SDS-PAGE and stained with PAS, no differences were observed in the protein composition irrespective of the different homogenization treatments applied (data not shown). Overall, this indicates that the conditions of homogenization did not affect the glycoprotein composition at the MFGM.

Oxidation. To evaluate the extent of lipid and protein oxidation in the fish oil enriched milk prepared under different conditions of homogenization PV, carbonyls (spectrophotometric and Western blot) and free thiol groups were measured. Multivariate data analysis was used to obtain an overview of all the data. Subsequently, the results obtained from each method were interpreted separately.

Multivariate Data Analysis on Oxidation Data. In the ANOVA PLSR analysis three principal components (PCs) were validated. The two first PCs explained most of the variation in the oxidation results. These two PCs explained 65% of the variance in X (design variables: pressure and temperature) and 60% of the variance in Y (measured variables: droplet size, PV, carbonyls, and free thiol groups). The third PC did not contribute further to explaining the relationship between samples and the measured variables than already observed for the two first PCs.

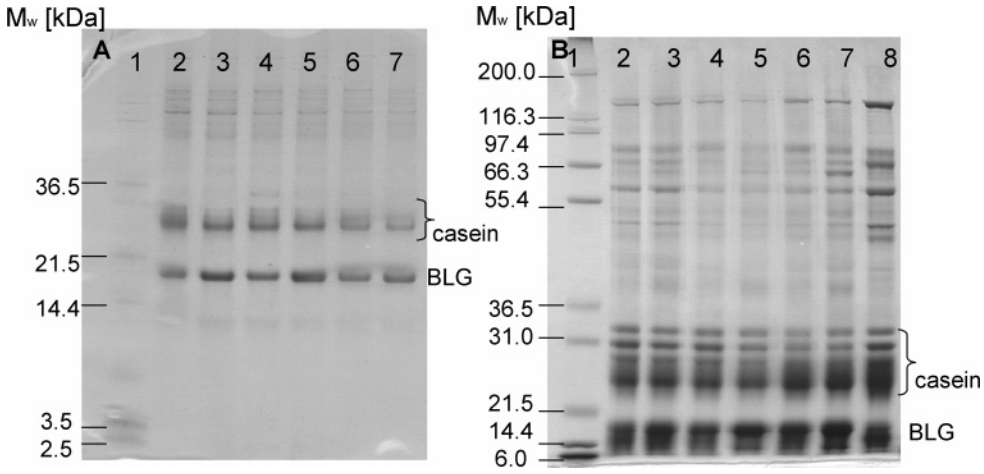

Figure 3. SDS-PAGE patterns, obtained under reducing conditions, of MFGM material washed fat from fish oil enriched milk produced under different conditions of homogenizations. Proteins were separated in MES (**A**) and MOPS (**B**) running buffer. Lanes: 1, molecular weight standard; 2–7, membrane material from milk emulsions produced at different conditions [(2) 50 °C and 5.0 MPa; (3) 72 °C and 5.0 MPa; (4) 50 °C and 15.0 MPa; (5) 72 °C and 15.0 MPa; (6) 50 °C and 22.5 MPa; (7) 72 °C and 22.5 MPa]; 8, reference milk (without fish oil).

Table 4. Relative Quantities of β-Lactoglobulin, κ-Casein, β-Casein, α_{s1}-Casein, and α_{s2}-Casein Isolated from the MFGM in Milk Emulsions Produced at Different Homogenization Conditions and Reference Milk (without Oil Added and No Further Homogenization)[a]

MFGM sample						
pressure (MPa)	temperature (°C)	β-lactoglobulin	κ-casein	β-casein	α_{s1}-casein	α_{s2}-casein
5	50	8.01	16.10	5.07	5.94	5.14
	72	10.17	15.94	3.67	4.28	4.09
15	50	9.36	14.97	4.34	5.21	5.08
	72	14.56	14.17	3.65	4.55	4.59
22.5	50	9.45	14.93	5.18	4.56	4.23
	72	13.72	15.86	4.78	3.68	3.74
reference	no treatment	4.92	17.95	5.74	5.18	4.19

[a] The amount is calculated from the protein separation using MOPS running buffer.

The loadings plot (**Figure 4**) showed that there was a positive correlation between droplet size and PV and a negative correlation between PV and free thiol groups. Generally, results from the carbonyl determinations were not well described by this model as carbonyls 8 and 14 were located near the origin of the plot. However, the location of carbonyl 0 diagonally to that of carbonyl 1, which was near the high-temperature design variable, respectively, indicated, that at day 0 low temperature (50 °C) increased the concentration, whereas at day 1 high temperature (72 °C) increased the concentration of protein carbonyls. The low pressure and temperature were located close to the droplet size and PV in the model, which indicated that these homogenization conditions resulted in increased PV and droplet size. In contrast, high temperature was located close to free thiol groups, indicating an increased level of free thiol groups, when high homogenization temperature was applied. In an additional storage experiment performed on fish oil enriched milk, positive correlations between the measured PV and five different volatiles [1-penten-3-one, 2-penten-1-ol, 2-(*trans*)-hexenal, 2,4-(*trans,trans*)-heptadienal, and 2,6-(*trans,cis*)-nonadienal] were observed (data not shown).

Regressions coefficients calculated in the ANOVA PLSR model showed a significant influence of the design variables on some of the measured variables (**Table 5**). Low homogenization pressure had a significant positive influence on the droplet size and PV, whereas high homogenization pressure had a significant negative influence on the droplet size. Regarding the homogenization temperature, high temperature had a significant negative influence on PV and a significant positive influence on free thiol groups.

Lipid Oxidation. The lipid oxidation, measured as PV, was in the range of 3.2−24.8 mequiv of peroxides/kg of oil (**Figure 5**) during the storage period (2 weeks). PVs increased in most of the milk emulsions during the storage period, but the increase in PV depended on the conditions of homogenization. In general, PVs in milk emulsions produced at 50 °C increased more rapidly compared to emulsions produced at higher temperature (72 °C). For milk emulsions produced at 72 °C and 15 or 22.5 MPa only a slight increase in PVs was observed. These findings confirmed the interpretation of the model based on the ANOVA PLSR.

Protein Oxidation. Progress in protein oxidation in the milk emulsions was monitored by development of protein carbonyls and changes in concentration of free thiol groups.

The amount of free thiol groups was measured at days 0 and 14 and ranged between 0 and 4.28×10^{-4} nmol/mg of protein (**Figure 6**). Milk emulsions produced at high temperature (72 °C) had higher concentrations of free thiol groups compared to emulsions produced at low temperature (50 °C). For milk emulsions produced at 5 MPa at both temperatures (50 and 72 °C) and the one produced at 15 MPa at 50 °C the thiol groups tended to increase during storage, whereas the concentration of thiol groups tended to decrease in the other emulsions.

The concentration of carbonyl groups increased in all samples during the 2 weeks of storage when the values for the start and end storage days were compared (**Table 6**). The concentrations ranged in this period between 0.2 and 6.0 nmol/mg of proteins. High pressure (22.5 MPa) had a low starting point, but the increase of carbonyls during storage was relatively highest for emulsions prepared at this pressure. However, there was no systematic pattern in the development of protein carbonyls measured spectrophotometrically in the different milk emulsions if all of the measured storage days were compared, as also shown in the APLSR plot (**Figure 4**).

The formation of protein carbonyls was also detected qualitatively in the milk emulsions by Western blot (**Figure**

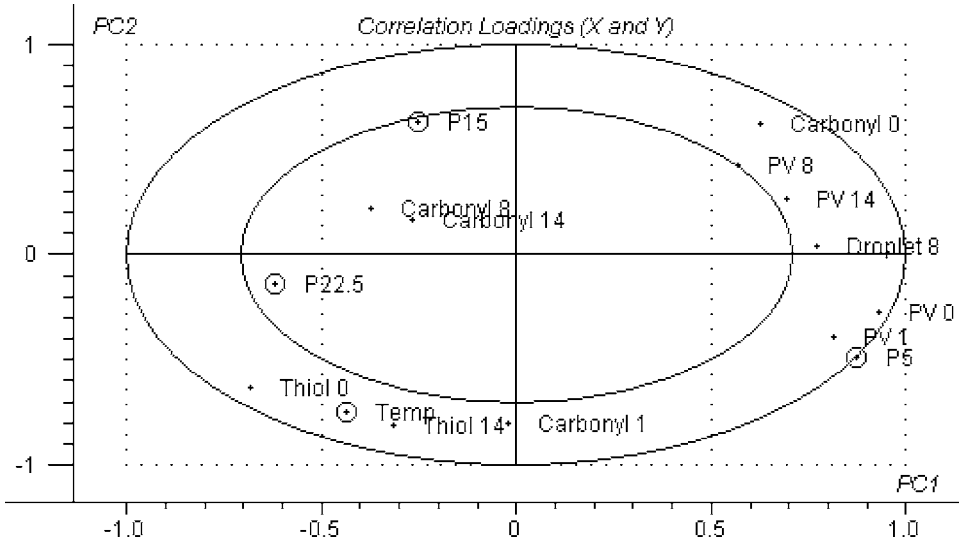

Figure 4. Loadings plot of PC1 versus PC2 from ANOVA PLSR analysis. Design variables (homogenization pressure and temperature) were used as *X* variables, and measured variables (droplet size, PV, carbonyls, and free thiol groups) were used as *Y* variables. Numbers after the variable names (thiol, PV, carbonyl) indicate storage time in days.

Table 5. Regression Coefficients Calculated in the ANOVA PLSR Model

	pressure (MPa)			
	5.0	15.0	22.5	temperature (72 °C)
droplet size	+		−	
PV	+			−
carbonyls				
thiol groups				+

[a] A significant positive influence is indicated by (+) and significant negative influence by (–).

Table 6. Levels [Average, Minimum, and Maximum ($n = 3$)] of Protein Carbonyls in the Different Milk Emulsions at Storage Days 0 and 14

milk emulsions		carbonyls (nmol/mg of protein)					
		storage day 0			storage day 14		
temperature (°C)	pressure (MPa)	av	min	mx	av	min	max
50	5	2.2	1.8	2.6	3.1	2.5	3.6
	15	2.4	2.3	2.5	6.0	5.7	6.4
	22.5	0.4	0.0	0.9	5.6	4.5	6.3
72	5	0.8	0.5	1.5	4.8	4.6	5.0
	15	1.0	0.3	1.7	6.1	4.5	7.5
	22.5	0.2	0.0	0.7	3.6	3.2	4.0

7). At the beginning of the storage, caseins seemed to be the only proteins that oxidized (**Figure 7B**). However, at day 11 a faint band appeared in all samples. This band represented a protein with a higher molecular mass than casein (**Figure 7C**), and the band was estimated to have a molecular mass of around 62−64 kDa. This may be BSA (66 kDa), a whey protein, or a membrane protein, such as butyrophilin (67 kDa). The data indicated that the amount of protein carbonyls increased during storage (**Figure 7A**), but it was not possible to observe differences between the different treatments either at day 0 or at day 11 (**Figure 7B**,**C**).

DISCUSSION AND CONCLUSIONS

The oil droplet size was affected by both the homogenization temperature and the pressure applied for incorporation of fish oil in milk. Increasing temperature and pressure reduced the size of the oil droplets. This is in agreement with an earlier study on fish oil enriched milk by Let et al. (*6*) and the general theory about homogenization pressure reducing the droplet size of emulsions (*11*).

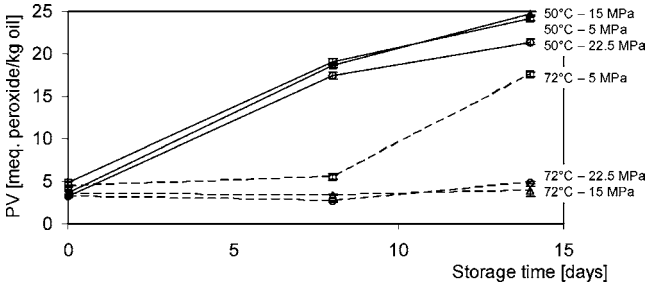

Figure 5. PV measured in the different milk emulsions at days 0, 8, and 14. The bars indicate the standard derivation ($n = 4$).

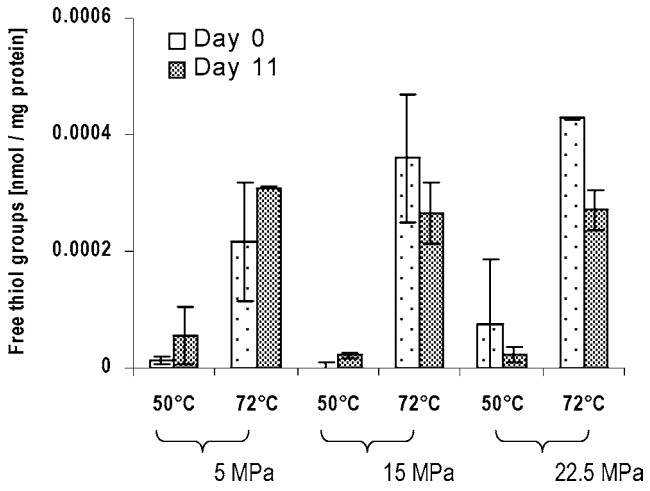

Figure 6. Concentration of free thiol groups (nanomoles per milligram of protein) in the milk emulsions after 0 and 11 days of storage. The error bars indicate the standard derivation ($n = 2$).

Changes in the Protein Composition of the Oil−Water Interface. The results obtained by SDS-PAGE and CLSM

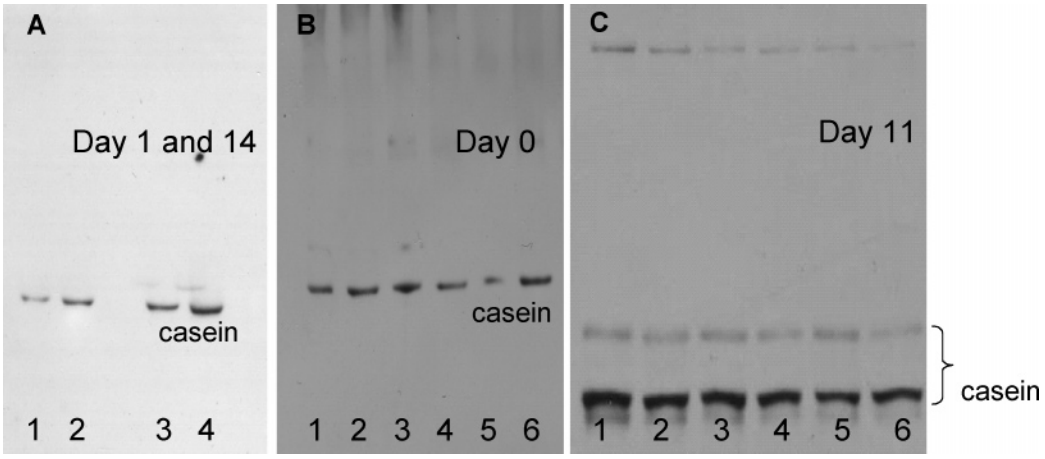

Figure 7. Detected protein carbonyls by Western blot. Proteins were separated in MES (**A**, **B**) or MOPS (**C**) running buffer. (**A**) Milk emulsions produced under different homogenization conditions. Lanes: (1) 72 °C and 15 MPa at day 1; (2) 72 °C and 15 MPa at day 14; (3) 50 °C and 22.5 MPa at day 1; (4) 50 °C and 22.5 MPa at day 14. Storage days 0 (**B**) and 11 (**C**). Lane: (1) 50 °C and 5 MPa; (2) 72 °C and 5 MPa; (3) 50 °C and 15 MPa; (4) 72 °C and 15 MPa; (5) 50 °C and 22.5 MPa; (6) 72 °C and 22.5 MPa.

indicated that the conditions of homogenization affected the protein composition at the oil−water interface. A higher amount of β-lactoglobulin and a lower amount of caseins at the oil−water interface were observed when the milk emulsion was homogenized at 72 °C compared to 50 °C. β-Lactoglobulin is a globular protein, which has a free thiol group hidden in its inner core (*11*). Unfolding of β-lactoglobulin due to heat treatment results in exposure of hydrophobic regions of the molecule, which enables β-lactoglobulin to adsorb to the MFGM (*11*, *30*). Therefore, the larger amount of β-lactoglobulin at the MFGM at higher temperature might be a consequence of the higher hydrophobicity of β-lactoglobulin after unfolding. Thereby, the free thiol group hidden in the core of β-lactoglobulin also becomes exposed, which is in agreement with the higher level of free thiol groups found at 72 °C compared to 50 °C in the present study. A previous study by Brun and Dalgleish (*31*) on milk protein adsorption to an oil−water interface indicated that β-casein and α_{s1}-casein were displaced by whey proteins as a consequence of heating. The present finding that concentrations of β-lactoglobulin increased when the homogenization temperature was increased from 50 to 72 °C was therefore in accordance with the results obtained by Brun and Dalgleish (*31*). Furthermore, the whey protein BSA also contains a free thiol group, which may have contributed to the measured concentration of free thiol group. The concentration of this protein is, however, much smaller compared to β-lactoglobulin (around 5 times) (*11*). Moreover, there were not observed any differences in the amount of BSA at the interface between the different emulsions. Therefore, the differences in the concentration of the free thiol groups in the different emulsions are suggested to be due to the differences in the concentration of β-lactoglobulin at the interface in the emulsions only.

PAS staining indicated no differences in the level of glycoproteins at the MFGM between the treatments applied. The homogenization process led to incorporation of significant amounts of β-lactoglobulin from the aqueous phase to the MFGM and maybe also in release of material from the membrane as also observed in other studies (*11*−*13*, *15*). Thereby, the possible changes in the composition of the original membrane glycoproteins are suggested to be masked by the large amount of attached proteins from the aqueous phase.

Effect of Homogenization Treatment on the Oxidation of Lipids and Proteins. The development of peroxides, which are primary lipid oxidation products, was grouped according to the homogenization temperature applied. Emulsions produced at 50 °C had a higher content of peroxides. The results showed that harsher homogenization treatments (72 °C and 15 or 22.5 MPa, respectively) resulted in emulsions that were more stable against lipid oxidation compared to milder conditions (50 °C and 5 or 15 MPa). This result confirmed earlier findings by Let et al. (*6*), who measured development of both primary and secondary lipid oxidation products during storage.

Two of the milk emulsions (50 °C and 15 MPa; 72 °C and 5 MPa) resulted in the same droplet size, but showed different oxidative stabilities. This finding is not in agreement with the hypothesis that there is a relationship between the droplet size and oxidative stability. The different developments in lipid oxidation obtained in emulsions with the same droplet size could indicate that other factors such as the composition of the oil−water interface played a major role.

The homogenization conditions did not significantly influence the development of protein carbonyls, which is one of the products formed during protein oxidation. The qualitative determination of carbonyl groups suggested that the caseins were the most exposed proteins with respect to formation of protein carbonyls. Decrease in the concentration of free thiol groups in the milk emulsions during storage might well be an indication of protein oxidation or denaturation. Development of protein carbonyl content during the storage of the milk emulsion was not easy to detect, and this might be due to the unspecificity of the DNPH assay and the inaccuracy of the methods. Moreover, severe oxidation can result in loss of amino acids, but amino acid analysis of the casein precipitated from the most oxidized milk emulsion (50 °C and 15 MPa) did not reveal any differences in amino acid composition during the entire storage period (data not shown). These results further confirm that protein oxidation even if detected during storage was not easy to quantify.

Protein Composition of the Oil−Water Interface versus Oxidation. The results indicated that less lipid oxidation was detected when the milk emulsion was produced at high temperature (72 °C) and pressure (22.5 MPa). Under these conditions of homogenization, the oil−water interface contained a higher level of β-lactoglobulin and less casein compared to the emulsion produced under other homogenization conditions. This might indicate that β-lactoglobulin acts as a physical barrier

at the interface between the oil inside the oil droplet and oxidation catalysts such as metal ions in the water phase. We also suggest that the interface is better covered with unfolded β-lactoglobulin than with intact casein micelles, which were present to a larger extent when homogenization was performed at low temperature and pressure. Less casein at the interface means more casein in the aqueous phase of the milk emulsion produced at 72 °C and 22.5 MPa compared to the emulsion produced under other conditions of homogenization. A recent study indicated that casein in the aqueous phase of algal oil enriched milk induced an increased oxidative stability of the milk due to the ability of caseins to act as a metal chelator in the aqueous phase, preventing oxidation at the interface (*18*). Therefore, an increase in casein in the aqueous phase together with an increased level of β-lactoglobulin at the interface is suggested to reduce lipid oxidation in milk enriched with n-3 PUFA. Moreover, the finding that lactoferrin formed aggregates with casein micelles at high homogenization temperature and pressure may also influence the ability of both lactoferrin and casein to chelate metal ions. This needs to be further investigated. In addition, the unfolded β-lactoglobulin at the oil−water interface had exposed free thiol groups, which may act as antioxidants as deduced from the observed decrease in the level of free thiol groups during storage.

In summary, the present study confirmed the previous findings that the best oxidative stability of the n-3 lipids was obtained when the fish oil enriched milk was homogenized at a high temperature (72 °C) and pressure (22.5 MPa). The high oxidative stability obtained using these conditions was most likely due to an optimal protein composition at the oil−water interface that gives the best protection against oxidation. Hence, the composition at the interface seemed to be very important, and it is demonstrated that thermal oxidation is not triggered by the high temperature used (72 °C), but rather that this high temperature results in unfolding of the proteins at the interface, which in turn gives the highest protection against oxidation.

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