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Changes in the surface protein of the fat globules during ultra-high pressure homogenisation and conventional treatments of milk

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ABSTRACT

Disruption of fat globules upon homogenisation provokes a reduction of their size and a concomitant increase in their specific surface area. In order to overcome this phenomenon, the milk fat globule membrane (MFGM) adsorbs non-native MFGM proteins. The aim of the present study was to examine the effects of UHPH conditions (temperature and pressure) on the milk fat globule and the surface proteins by comparison with conventional treatments applied in the dairy industry. Transmission electron microscopy and SDS-PAGE revealed major differences. In UHPH-treated milk, casein micelles were found to be adsorbed on the MFGM in a lesser extent than in conventional homogenisation –pasteurisation. However, greater adsorption of directly bonded casein molecules, released by UHPH through the partial disruption of casein micelles, was observed especially at high UHPH pressures. Adsorption of whey proteins on the MFGM of conventionally homogenised–pasteurised milk was mainly through intermolecular disulfide bonds with MFGM material, whereas in UHPH-treated milk, disulfide bonding with both indirectly and directly adsorbed caseins was also involved.

1. Introduction

Milk fat globules are surrounded by a membrane composed mainly of proteins, phospholipids, glycoproteins, triglycerides, cholesterol and enzymes. This membrane, which is known as the milk fat globule membrane (**MFGM**), consists of several distinct layers of different origins. Precursors of milk lipid globules are formed at the endoplasmic reticulum of mammary epithelial cells and are released into cytosol as lipid droplets surrounded by a monolayer coat of proteins and phospholipids. During secretion, milk fat globules gain the outer bilayer coat from the apical plasma membrane of secretory cells (Keenan & Mather, 2006).

The protein composition of the MFGM is very complex with over 40 different polypeptides, ranging in molecular weight from 15 to 240 kDa (Mather, 2000). The major MFGM proteins are xanthine oxidase (**XO**; 155 kDa), butyrophilin (**BTN**; 67 kDa), and PAS 6/7 (49–50 kDa) (Mather, 2000). Since cross-links between MFGM proteins, e.g. XO-BTN, through intermolecular disulfide bonds occur naturally, the protein composition of the MFGM is usually studied with 8% polyacrylamide SDS-PAGE gels under reducing conditions. Previous studies have shown that milk processing, i.e., heating (Ye, Singh, Taylor, & Anema, 2002; Ye, Singh, Taylor, & Anema, 2004a), evaporation (Ye, Singh, Taylor, & Anema, 2004b), and homogenisation (Ye, Anema, & Singh, 2008), does not provoke major changes on the composition of native MFGM proteins.

However, milk processing provokes interactions between MFGM components and whey proteins and/or caseins (Ye et al., 2004a,b, 2008). Since whey proteins and caseins run out of 8% polyacrylamide gels due to their low molecular masses (~14–25 kDa), resolving gels of 15% polyacrylamide are usually used to study them. In addition, interactions between MFGM and caseins might be through adsorption of casein micelles (indirect adsorption) at the fat globule surface or through direct adsorption and/or bonding of the protein molecules. In order to differentiate direct from indirect adsorption, urea–ethylenediamine-tetraacetic acid (**EDTA**) buffer is used; washing isolated MFGM material with the buffer dissociates and washes away the casein micelles adsorbed at the fat globule surface, but protein molecules adsorbed directly at the interface of fat globules and the protein molecules bound to the interfacial protein layer via covalent bonds remain on the surface of the fat globule (Ye et al., 2004b).

A previous work about the effect of ultra-high pressure homogenisation (**UHPH**) on rennet coagulation properties (Zamora, Ferragut, Jaramillo, Guamis, & Trujillo, 2007) showed that

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UHPH enhances the coagulation properties of milk, and such improvement was attributed to changes at the protein-fat structures. Moreover, fat content of milk was proven to greatly influence the protein–protein interactions within rennet curds from UHPH-treated milk (Zamora, Armaforte, Trujillo, & Kelly, 2008). From a technological point of view, UHPH triggered textural changes in fresh cheeses, i.e., higher firmness, lower deformability and higher water retention, which could be explained by the reduction of fat globule size and the incorporation of caseins and whey proteins at the milk fat globule membrane (Zamora, Ferragut, Juan, Guamis, & Trujillo, 2011). Thus, the aim of the present study was to examine the effects of UHPH conditions (temperature and pressure) on the milk fat globule and the surface proteins.

2. Material and methods

2.1. Milk supply and treatment

Raw whole bovine milk was obtained from a local dairy farm (S.A.T. Can Badó, Roca del Vallès, Spain). Milk was standardised at 3.6% fat and kept overnight at 4 °C. Before all treatments, the milk was warmed to approximately 20 °C. Single-stage UHPH was carried out at 100, 200 and 300 MPa with inlet temperatures (T_i) of 20, 30 and 40 °C using the modified model FPG11300 of Stansted Fluid Power Ltd. (model FPG11300, Stansted Fluid Power Ltd., Harlow, UK). UHPH equipment comprised a feeding tank and a heat-exchanger, keeping the milk at the desired T_i, followed by the high-pressure system, with a Stansted valve preceding a refrigeration system, and a cooling system consisting of two heat-exchangers.

Milk temperature during UHPH treatment was measured with thermocouples located at different points of the equipment. Real inlet temperature (T_{in}) corresponded to milk temperature at the exit of the first heat-exchanger. Milk temperature before and immediately after the Stansted valve were referred as T_1 and T_2. The refrigeration system located behind the valve cooled down milk to T_r. Outlet temperature (T_{out}) was measured after the cooling system.

UHPH was compared with conventional treatments, i.e., pasteurisation (**PA**; 72 °C for 15 s) and homogenisation–pasteurisation (**HP**; 15 + 3 MPa at 60 °C, 72 °C for 15 s), carried out with a Finamat heat-exchanger (model 6500/010, GEA Finnah GmbH, Ahaus, Germany), and a Niro Soavi homogeniser (model X68P Matr. 2123, Niro Soavi, Parma, Italy).

2.2. Mean diameter and specific surface area of fat globules

Determination of the surface-weighted mean diameter [D(3,2)] and the specific surface area (SSA) was carried out using a Beckman Coulter laser diffraction particle size analyser (LS 13,320 series, Beckman Coulter, Fullerton, CA, USA) as described by Zamora et al. (2007).

2.3. Isolation of MFGM material

MFGM components were obtained following the method of Ye et al. (2002) with modifications. Cream was separated by centrifugation at 10,500 × *g* for 30 min at 20 °C after addition of 28.6 g of sucrose 100 g^{-1} of milk in order to increase the difference in density between fat and serum phases (Cano-Ruiz & Richter, 1997). After cooling, the top layer (cream) was removed from the centrifuge tube using a spatula. Cream was washed twice for 1 h at room temperature in 10 volumes of simulated milk ultrafiltrate (**SMUF**; Jenness & Koops, 1962) or SMUF containing 6 M urea and 50 mM EDTA (Ye et al., 2002), centrifuged at 10,500 × *g* for 30 min at 20 °C and solidified by cooling at 4 °C in order to remove the top layer, i.e., washed cream.

2.4. Determination of washed cream protein content

Total protein content of the washed creams was determined in triplicates through the Dumas combustion method (IDF, 2002) by determining total nitrogen and multiplying by a factor of 6.38. Protein coverage was calculated by dividing the amount of protein per gram washed cream by the SSA of fat globules (Lee & Sherbon, 2002).

2.5. Analysis of MFGM protein components

Protein composition of MFGM from washed creams was determined by SDS-PAGE. Samples of washed creams (0.25 g) were dispersed in 0.5 mL Tris–HCl buffer (6% Tris 0.5 M, 10% glycerol, 2% SDS and 0.05% bromophenol blue). For non-reducing PAGE, the samples were heated at 45 °C for 5 min. For reducing conditions, 5% β-mercaptoethanol was added to the samples before heating at 95 °C for 5 min in a boiling water bath. In order to remove fat, the samples were centrifuged at 2500 × *g* for 30 min. 15 μL of supernatants diluted in sample buffer (1:3 v/v) were loaded onto 15% SDS-polyacrylamide gels (37.5% Acryl-Bis at 40% in 1.5 M Tris–HCl buffer, pH 8.8, for separating gel; 10% Acryl-Bis at 40%, in 0.5 M Tris–HCl buffer, pH 6.8, for stacking gel). Molecular mass markers of 212, 116, 97, 66, 45, 31, 21, 20 and 14 kDa (Amresco, Solon, USA) and milk protein standards, i.e., bovine serum albumin, caseins (**CN**), α-lactalbumin (**α-LA**) and β-lactoglobulin (**β-LG**) (Sigma–Aldrich Chemie, Steinheim, Germany) were applied to each gel. Gels were run at 200 V using a Pharmacia Biotech power supply unit (model EPS 3500, Pharmacia Biotech, Uppsala, Sweden). Protein bands were stained with a solution of Coomassie brilliant blue R-250. Gels were destained with a solution of methanol and glacial acetic acid at concentrations of 160 and 10 mL L^{-1}, respectively. Scanned images of the destained gels were analysed using the ImageMaster software (Amersham Pharmacia Biotech, Newcastle, UK). The apparent molecular mass (*Mr*) of the bands on the SDS-PAGE was estimated from the mobility of proteins in the gel when compared with the mobility of the molecular mass markers. Protein identification was carried out by comparison with protein standards, for caseins and whey proteins, and with results from previous studies for MFGM proteins (Singh, 2006; Ye et al., 2002, 2004b).

2.6. Transmission electron microscopy

Microstructure of milk was observed by transmission electron microscopy (TEM). Milk samples were mixed with glutaraldehyde (3% final concentration) in a bijou bottle and then mixed with warm 2% low-temperature gelling agar at a 1:1 ratio. The mixture was allowed to gel and was chopped into 1 mm^3 cubes. The cubes were then washed as follows: with 0.1 M sodium cacodylate buffer pH 7.2 for 30 min, then again twice for 1 h, with 1 mL of a solution containing 50% osmium tetroxide (2% solution) and 50% cacodylate/HCL buffer for 2 h, with 1 mL of % uranium acetate for 30 min, and finally with water. Dehydration consisted of washing with ethanol at increasing concentration (50–100%) and time (5–60 min). The ethanol was poured off and the bottle was filled with incomplete resin [20 mL epoxy resin, 20 mL dodecylsuccinic anhydride (DDSA) and 1 drop of dibutyl phthalate] and placed on a rotator overnight. Incomplete resin was replaced with complete resin [incomplete formulation with addition of 0.6 mL of the plasticiser benzyldimethylamine (BDMA)] and then placed on the rotator for 4 h. Sample cubes were added to moulds containing fresh complete