Filtration of Milk Fat Globule Membrane Fragments from Acid Buttermilk Cheese Whey

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ABSTRACT

The proteins and polar lipids present in milk fat globule membrane (MFGM) fragments are gaining attention for their technological and nutritional properties. These MFGM fragments are preferentially enriched in side streams of the dairy industry, like butter serum, buttermilk, and whey. The objective of this study was to recover MFGM fragments from whey by tangential filtration techniques. Acid buttermilk cheese whey was chosen as a source for purification by tangential membrane filtration because it is relatively rich in MFGM fragments and because casein micelles are absent. Polyethersulfone and cellulose acetate membranes of different pore sizes were evaluated on polar lipid and MFGM-protein retention upon filtration at 40°C. All fractions were analyzed for dry matter, ash, lipids, proteins, reducing sugars, polar lipid content by HPLC, and for the presence of MFGM proteins by sodium dodecyl sulfate-PAGE. A fouling coefficient was calculated. It was found that a thermocalcic aggregation whey pretreatment was very effective in the clarification of the whey, but resulted in low permeate fluxes and high retention of ash and whey proteins. By means of an experimental design, the influence of pH and temperature on the fouling and the retention of polar lipids (and thus MFGM fragments), proteins, and total lipids upon microfiltration with 0.15 μ M cellulose acetate membrane was investigated. All models were highly significant, and no outliers were observed. By increasing the pH from 4.6 to 7.5, polar lipid retention at 50°C increased from 64 to 98%, whereas fouling of the filtration membrane was minimized. A 3-step diafiltration of acid whey under these conditions resulted in a polar lipid concentration of 6.79 g/100 g of dry matter. As such, this study shows that tangential filtration techniques are suited for the purification of MFGM fragments.

Key words: acid buttermilk cheese whey, milk fat globule membrane, microfiltration, polar lipid

INTRODUCTION

Whey, the by-product of cheese and casein manufacturing, contains 0.4 to 0.5% of residual fat (Rombaut et al., 2007). This lipid fraction is the main cause of whey turbidity and consists of small fat globules, lipoprotein particles, and milk fat globule membrane (MFGM) fragments. The MFGM is a true biological bilayer of polar lipids (PL) and proteins that surrounds the fat globules in raw milk, keeping them in emulsion and protecting them from enzymatic attack (Danthine et al., 2000). About two-thirds of the MFGM are membrane-specific proteins, of which xanthin oxidase/dehydrogenase, adipophilin, butyrophilin, CD 36, and periodic acid/Schiff 6/7 are the most abundant (Mather, 2000). The PL fraction of the MFGM consists mainly of phospholipids like phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, and phosphatidylinositol, and sphingolipids like sphingomyelin and gluco- and lactosylceramide (Keenan et al., 1999). The MFGM proteins and PL are closely associated with each other and exist in the membrane as complexes, often covalently linked (Deeth, 1997). As such, during processing of milk, the MFGM is ruptured and substantial amounts of vesicle-like membrane material migrate to aqueous phases like buttermilk, butterserum (the aqueous phase of butter), and whey. Therefore, whey contains up to 14 to 22 mg of phospho- and sphingolipids per 100 g (Rombaut and Dewettinck, 2006).

Due to the amphiphilic nature of PL and membrane proteins, MFGM material possesses good emulsifying capacities (Kanno et al., 1991; Roesch et al., 2004). The MFGM isolates could be applied as emulsifier in a whole range of products. In addition to their technological properties, some MFGM constituents are highly bioactive components, which have profound effects on cell metabolism and regulation. Especially sphingolipids are gaining attention, as evidence about their positive health effects is accumulating. In tests on mice in which tumorigenesis was induced by a chemical agent or caused by an inherited genetic defect, sphingolipids were found to inhibit the early and late stages of colon carcinogenesis (Schmelz et al., 1996; Schmelz, 2004).

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Dietary sphingolipids were also found to significantly lower cholesterol absorption (Eckhardt et al., 2002; Noh and Koo, 2004) and to retard the growth of potentially harmful bacteria in the colon (Rueda et al., 1998). Besides sphingolipids, MFGM proteins are believed to exert health-improving properties. Peptides of these proteins can positively interfere in breast and colon cancer, multiple sclerosis, and hypercholesterolemia (Spitsberg, 2005).

For the separation of MFGM fragments, tangential filtration is convenient and straightforward, provided that no casein micelles are present. Casein micelles and MFGM fragments are roughly similar in size and are therefore virtually impossible to separate by filtration techniques. Because whey is free of caseins, the MFGM material can easily be concentrated by filtration, even if its MFGM concentration is limited compared with other dairy fractions like buttermilk or butter serum. Thermocalcic aggregation (TA), which is a sequestration of residual lipids, lipoproteins, and MFGM fragments by calcium addition and moderate heating, could be useful for purification of MFGM material, although it was originally developed for clarification of the whey (Maubois et al., 1987). After coagulation, these aggregates can be separated by settling, filtration, or centrifugation. This process is applied in the manufacturing of whey protein concentrates as pretreatment prior to UF because residual fat seriously alters the functionality of whey protein concentrates and is also the main cause for rapid fouling and flux decline of the UF membrane (Fauquant et al., 1985; Pierre et al., 1992). To purify phospholipids, Sachdeva and Buchheim (1997) performed a combination of UF, microfiltration (MF), and diafiltration without any pretreatment on acid and rennet buttermilk cheese whey. They achieved a 10-fold increase in PL expressed on DM but obtained varying phospholipid recoveries and low permeate fluxes. Processing parameters like temperature or pH were not investigated. These 2 parameters can, however, seriously change filtration characteristics like permeate flux, fouling of the membrane, retention coefficients, and recoveries of each whey component and thus influence the composition of the final purified fraction (Daufin et al., 1991; Cheryan, 1998; Rao, 2002).

This study deals with the effect of membrane type, membrane pore size, and whey pretreatment on the filtration characteristics and MFGM material retention and recovery upon filtration of acid buttermilk cheese whey. The influence of temperature and pH is investigated, as well as the possibility of diafiltration to further concentrate PL.

MATERIALS AND METHODS

Materials

Acid buttermilk cheese whey was obtained from a local dairy company (Büllinger Buttereier, Büllingen, Belgium). Here, the buttermilk was acidified with a starter culture and separated with a quark centrifuge in quark and whey (Boone, 2003). All reagents used during processing and analysis were of 98+ purity. Chloroform, methanol, triethylamine, and formic acid used as mobile phase for HPLC analysis of PL were of HPLC grade. A milk PL standard was supplied by Spectral Services (Köln, Germany).

Analysis

DM, Ash, Protein, Total Lipids, Reducing Sugars, PL, and Optical Density. The DM analyses were performed by gravimetric difference after heating (IDF, 2004). Ash content was determined by gravimetric difference after ashing following the AOAC method 945.46. Total lipid content was measured gravimetrically after evaporation of the extraction solvent using the Röse-Gottlieb extraction method (IDF, 1986). The protein content was obtained using the Kjeldahl nitrogen determination method (IDF, 1993), using 6.38 as the protein conversion factor. Sugar content was determined by measuring the amount of reducing sugars (Luff Schoorl method; Acker, 1969). Polar lipid extraction and analysis by HPLC (Thermo Finnigan Surveyor, Thermo Corp., Brussels, Belgium) coupled to an evaporative light scattering detector (ELSD 2000, Alltech Inc., Lokeren, Belgium) were performed as described elsewhere (Rombaut et al., 2005). All values were expressed in weight percentage, unless stated otherwise. The optical density of the permeates was measured with an absorbance spectrophotometer (Varian, Cary 500, Sint-Katelijne-Waver, Belgium) at 590 nm with ultrapure water as reference. All analyses were performed in duplicate.

Protein Profiling by SDS-PAGE. All reagents and precast gels were purchased from Invitrogen (Merelbeke, Belgium). The samples were prepared following the manufacturer's protocol. Two hundred fifty microliters of NuPAGE LDS sample buffer and 100 μ L of NuPAGE reducing agent were added to 650 μ L of sample. The samples were vortexed and heated at 70°C for 10 min in a hot water bath. Ten microliters of each sample was loaded onto a NuPAGE bis-tris 4 to 12% polyacrylamide gel (dimension: 10 $\times$ 10 $\times$ 0.15 cm). The MOPS buffer (3-N-morpholinopropanesulfonic acid) was used as the running buffer. Separation was performed in 55 min at 200 V and an initial current of 125 mA/gel. After separation, the gel was washed 3 times with deionized

water and stained for 60 min with Simply Blue safestain (Coomassie blue). The gel was destained for 60 min in deionized water and further overnight in 100 mL of a 3.3% NaCl solution to increase staining intensity. Scanning of the wet gel was done with a high-resolution transmission scanner (UMAX Powerlook III, Taipei, Taiwan). Gels were analyzed with Imagemaster Total software (Amersham Biosciences, Roosendaal, the Netherlands).

The MFGM protein standard was purified from fresh raw milk, which was obtained from a local farmer. Ten liters of milk was decreamed at 42°C with a small continuous separator (Westfalia Separator, Oelde, Germany) at 12,000 rev/min, and the cream was washed 3 times with 9 L of a 1.5 g/L solution of KCl, to remove caseins and whey proteins. The washed cream was churned with a household beater for 10 min. The obtained butter was melted at 60°C, and an equal quantity of distilled water was added. The resulting slurry was centrifuged in a batch lab centrifuge (Martin Christ Osterode, Harz, Germany) at $2,400 \times g$ for 5 min at 60°C, and the butter serum was collected. Butter serum and buttermilk were pooled and freeze dried. To remove the lipids, the powder was washed with chloroform, filtered on a paper filter, and allowed to dry. The purified MFGM protein powder was stored at -32°C. Classification of the MFGM proteins was done according to the review article of Mather (2000).

Processing

Filtration of Acid Buttermilk Cheese Whey. The UF unit existed of a Millipore Pellicon 2 lab scale plate and frame filtration unit equipped with a polyethersulfone (PES) membrane with nominal cut-off value of 30 kDa (Millipore, Brussels, Belgium). The MF unit existed of an Optisep 200 flatbed filtration module (Cary, NC). The membranes were made from cellulose acetate (CA) with a pore diameter of 0.15, 0.2, and 0.45 μm and from polyethersulfone with a pore diameter of 0.1 and 0.2 μm (Schleicher and Schuell, Gent, Belgium). The acid buttermilk cheese whey was pumped by an Almatec diaphragm pump connected with a pulse dampener (Kamp-Lintfort, Germany). The transmembrane pressure (TMP) was kept constant at 0.1 MPa. The feed flow was 500 L/h. For each MF process, a new membrane of the same batch was taken to ensure that observed differences in filtration behavior were not caused by incomplete membrane cleaning. Prior to filtration, the membrane was flushed with 10 L of deionized water. The pH of the acid buttermilk cheese whey was adjusted with a HCl (1 N) or KOH (1 N) solution. The temperature of the whey was carefully controlled by using a hot water bath. During startup, the TMP

was gradually increased (0.01 MPa/min) to 0.1 MPa by partially closing the retentate valve. Ten liters of acid buttermilk cheese whey was filtered to the desired volume concentration ratio (VCR). After filtration, the membrane was flushed with 10 L of deionized water. Samples of original acid buttermilk cheese whey, retentate, and permeate were stored at -32°C prior to analysis. The retention coefficient (RC; %) was calculated using the equation $\text{RC}(\%) = [(1 - (C_p/C_r)) \times 100]$, where C_p and C_r are the concentrations (g/100 g) of a component in the permeate and retentate, respectively.

Thermocalcic Aggregation. Thermocalcic aggregation (TA) of MFGM fragments was performed as described by Maubois et al. (1987). In brief, 2.2 g of CaCl_2 /L whey was added to 10 L of whey at 2°C. The pH was adjusted to 7.3, and the whey was heated rapidly to 50°C and kept at this temperature for 8 min, allowing the calcium phosphate complexes to be formed.

Fouling Coefficient. A fouling coefficient (FC) was adapted from Rao (2002). For pure water, the permeate flux can be written as $J = \text{TMP}/(\mu_w \times R)$, where J is the permeate water flux, R is the hydraulic resistance, TMP is the transmembrane pressure, and μ_w is the dynamic viscosity of water (Cheryan, 1998). Prior to whey processing, the water flux after rinsing of the membrane was measured at a transmembrane pressure of 0.04, 0.06, 0.08, and 0.1 MPa. By linear regression, these data were fit to $J = a \times \text{TMP}$, where a is the slope of the curve. After filtration of whey, the membrane was rinsed again, and the water flux of the fouled membrane was determined by the same procedure and fitted to $J_{\text{fouled}} = a_{\text{fouled}} \times \text{TMP}$, where J_{fouled} is the permeate water flux after filtration and rinsing. The FC was calculated as $\text{FC} = 1 - (a_{\text{fouled}}/a)$.

Statistics and Experimental Design

Graphics were generated with Sigmaplot 6.0 (SPSS, Chicago, IL). Statistics and experimental design were performed with SPSS 12.0 (SPSS) and Design Expert 5.0 (Minneapolis, MN).

RESULTS AND DISCUSSION

Characterization of Whey

The whey (pH 4.57) used for the filtration experiments was the by-product of buttermilk quark manufacturing. The composition is given in Table 1. The PL was about 5 times higher than normal whey, which makes this fraction highly suitable as a source of MFGM material. Major PL were phosphatidylethanolamine (30.7 ± 1.6 g/100 g of PL), phosphatidylcholine (25.8 ± 1.7 g/100 g of PL), and sphingomyelin (21.9 ± 1.0 g/100 g of PL). Minor PL were phosphatidylinositol

Table 1. Whey composition (g/100 g $\pm$ SD)

	DM	Ash	Protein	Reducing sugars	Lipids	Polar lipids
Whey, g/100 g	5.64 $\pm$ 0.17	0.62 $\pm$ 0.02	0.47 $\pm$ 0.02	3.37 $\pm$ 0.2	0.39 $\pm$ 0.01	0.099 $\pm$ 0.001

(9.4 $\pm$ 1.4 g/100 g of PL), phosphatidylserine (6.7 $\pm$ 0.4 g/100 g of PL), lactosylceramide (4.5 $\pm$ 0.1 g/100 g of PL), and glucosylceramide (1.1 $\pm$ 0.2 g/100 g of PL). Total sphingolipid content accounted for 27.4 $\pm$ 1.0 g/100 g of PL. The SDS-PAGE revealed the presence of major amounts of MFGM proteins (Figure 1, lane 9). The presence of xanthin oxidase/dehydrogenase, CD 36, adipophilin, and periodic acid/Schiff 6/7 were affirmed by comparison with an MFGM protein reference (Figure 1, lane 10). Major and minor fractions of proteose peptone 3 with estimated MW of 25 and 16 kDa were observed. The broadness of the band was caused by the heavy glycosylation of the protein. Although still under some debate, this fraction was also considered as part of the MFGM protein fraction (Nejjar et al., 1990; Sorensen et al., 1997). Major whey proteins like BSA, β -LG A and B (Mailliart and Ribadeau-Dumas, 1988; Ebeler

et al., 1990), and α -LA were also identified. Caseins, with an electrophoretic mobility between 28 and 35 kDa, were not observed in the whey. These SDS-PAGE results should only be interpreted qualitatively and for comparison of the different fractions because the intensity of coloration of proteins is highly dependent on the type of protein. Moreover, saturation occurs very rapidly, resulting in a huge underestimation of the major proteins (our unpublished results).

Effect of Membrane Type, Pore Size, and Whey Pretreatment

Filtration Experiments. Acid buttermilk cheese whey was filtrated under 8 different conditions. All filtrations experiments were carried out with 10 L of whey, at 40°C, with a TMP of 0.1 MPa, a feed flow of 500 L/h and to a VCR of 5 (2 L of retentate, 8 L of permeate). Because MFGM fragments vary in size from 0.4 to 1.5 μ m (Corredig and Dalgleish, 1997), mainly MF membranes were used. The choice of membrane material (CA and PES) was module-limited and based on its difference in polarity (CA = hydrophilic; PES = hydrophobic). Three PES membranes were evaluated: 1 UF membrane with a 30-kDa MW cut-off and 2 MF membranes with 0.1- and 0.2- μ m pore diameter. Next to this, 3 CA MF membranes with 0.15-, 0.2-, and 0.45- μ m pore diameters were examined. Finally, MF was performed on a 0.15- and 0.45- μ m CA membrane with whey in which previously the MFGM fragments were sequestered by a thermocalcic aggregation pretreatment (Table 2). In Figure 2, the respective filtration curves are presented. All fluxes showed a similar pattern. First, there is a rapid drop of permeate flux, which is due to the formation of a concentration polarization layer and initial fouling, followed by a slow decrease in flux, caused by the increase of DM content of the feed and further fouling of the membrane. The flux was dependent on membrane pore size and material. The bigger the pore size, the higher the flux. Permeate fluxes were highest for CA membranes because these are more hydrophilic than PES membranes. A thermocalcic pretreatment of the whey resulted in very low permeate fluxes. Unexpectedly, after application of a thermocalcic aggregation treatment, the 0.45- μ m CA experiment exhibited a lower permeate flux than the 0.15- μ m CA experiment. When looking at the fouling coefficients in Table 2, heavy fouling occurred for all experiments,

Figure 1. The SDS-PAGE of butterserum whey permeates, obtained with different membranes. Lane 1: 30-kDa polyethersulphone (PES); lane 2: 0.1- μ m PES; lane 3: 0.15- μ m cellulose acetate (CA); lane 4: 0.2- μ m CA; lane 5: 0.2- μ m PES; lane 6: 0.45- μ m CA; lane 7: 0.15 CA + thermocalcic aggregation (TA); lane 8: 0.45 CA + TA; lane 9: acid buttermilk cheese whey; lane 10: milk fat globule membrane (MFGM) standard; lane 11: molecular weight marker. Observed MFGM proteins are adipophilin (ADPH), cluster of differentiation 36 (CD36), periodic acid Schiff 6/7 (PAS 6/7), proteose peptone 3 fraction 1 and 2 (PP3), and xanthin oxidase/dehydrogenase (XO). Observed whey proteins are BSA, immunoglobulin heavy chain (IGH), α -LA, and β -LG genetic variant A and B. Butyrophilin (BTN), one of the main MFGM proteins, was not observed.

Table 2. Fouling coefficient and characteristics ($\pm$ SD) of filtration permeates for different filtration experiments of acid buttermilk cheese whey¹

Experiment	Fouling coefficient	PL/DM in retentate (g/100 g)	Permeate optical density (λ_{590})	Permeate MFGM protein relative intensity (%)	β -LG and α -LA permeate SDS-intensity ratio
Whey		1.77 $\pm$ 0.06	3.056 $\pm$ 0.012	54.8 $\pm$ 0.3	1.78 $\pm$ 0.02
1) 30-kDa PES	0.80 $\pm$ 0.06	4.65 $\pm$ 0.14	0.002 $\pm$ 0.001	0.0 $\pm$ 0.0	0.93 $\pm$ 0.03
2) 0.1- μ m PES	0.95 $\pm$ 0.06	1.90 $\pm$ 0.11	0.431 $\pm$ 0.001	48.0 $\pm$ 0.3	1.81 $\pm$ 0.10
3) 0.15- μ m CA	0.96 $\pm$ 0.04	2.23 $\pm$ 0.09	0.834 $\pm$ 0.005	47.5 $\pm$ 1.1	1.76 $\pm$ 0.10
4) 0.2- μ m CA	0.91 $\pm$ 0.09	1.55 $\pm$ 0.07	1.008 $\pm$ 0.016	51.3 $\pm$ 2.2	1.72 $\pm$ 0.22
5) 0.2- μ m PES	0.84 $\pm$ 0.14	1.60 $\pm$ 0.09	1.014 $\pm$ 0.008	48.6 $\pm$ 0.4	1.86 $\pm$ 0.16
6) 0.45- μ m CA	0.91 $\pm$ 0.06	1.61 $\pm$ 0.12	1.682 $\pm$ 0.008	49.4 $\pm$ 1.3	1.75 $\pm$ 0.28
7) 0.15- μ m CA + TA	0.36 $\pm$ 0.01	4.65 $\pm$ 0.08	<0.001	0.0 $\pm$ 0.0	1.34 $\pm$ 0.06
8) 0.45- μ m CA + TA	0.86 $\pm$ 0.07	4.59 $\pm$ 0.04	<0.001	0.0 $\pm$ 0.0	1.29 $\pm$ 0.01

¹PL = polar lipids; MFGM = milk fat globule membrane; PES = polyethersulfone; CA = cellulose acetate; TA = thermocalcic aggregation.

except for the experiment with the 0.15- μ m CA membrane combined with a TA (experiment 7). In the latter experiment, a significantly lower degree of fouling was observed, which explains the higher flux compared with the experiment with the 0.45- μ m CA membrane combined with a TA (experiment 8). Most likely, in the latter case, particles were small enough to enter the membrane pores, leading to rapid and substantial fouling. The fact that with the 0.15- μ m CA membrane the

permeate flux was lowered after a TA, but nevertheless a low fouling coefficient was maintained, indicated the formation of a reversible polarization concentration layer, rather than actual fouling.

Filtration Efficiency. In Table 3, the retention coefficients of all components are given. The retention coefficients of the experiment with the 30-kDa PES membrane (experiment 1) and both experiments with a thermocalcic aggregation pretreatment (experiments 7 and 8) clearly differ from those of the other filtration experiments. In these 3 experiments, the permeates were clear, whereas the optical density of the permeates of the other experiments varied from 0.4 to 1.7 (Table 2). The retention of MFGM fragments in these 3 filtration experiments is much higher than in the other filtration experiments. The retention of PL tends toward 100%, and no MFGM fragments can be noticed in the permeates (Table 2 and Figure 1; lanes 1, 7, and 8), whereas in the other experiments, this is certainly not the case. As such, in these 3 experiments, the PL content expressed on DM (Table 2), which represents the degree of purification of MFGM fragments, increased to 4.6%. However, the retention of protein (and inherently the retention of DM) is also increased to >70% in these experiments. Significant correlations ($P < 0.05$) were found among polar lipid concentration, MFGM protein relative SDS-PAGE intensity, and the optical density, making the latter a good parameter for the removal of MFGM fragments. No significant differences in mass balances between different experiments were observed. Mass balance recovery of protein, lipids, and PL were from 86 to 99%, 73 to 93%, and 77 to 92%, respectively. This was also observed by other workers and indicates that these components make part of the fouling layer (Theodet and Gandemer, 1994; Rombaut et al., 2006).

In Table 2, the β -LG/ α -LA SDS-PAGE intensity ratio is given for all permeates and the whey. For the 30 kDa PES experiment (experiment 1) and both tests with

Figure 2. Permeate flux during the filtration of acid buttermilk cheese whey (pH 4.46), using different membranes: $\square$ 0.45- μ m cellulose acetate (CA), $\blacksquare$ 0.2- μ m CA, ∇ 0.15- μ m CA, $\blacktriangledown$ 0.2- μ m polyethersulfone (PES), $\blacklozenge$ 0.15 μ m-CA + thermocalcic aggregation treatment, $\circ$ 0.1- μ m PES, $\diamond$ 0.45- μ m CA + thermocalcic aggregation treatment, $\bullet$ 30-kDa PES. Temperature was kept at 40°C, the transmembrane pressure at 0.1 MPa and the feed velocity over the membrane at 2.14 m/s.

Table 3. Retention coefficients (% $\pm$ SD) upon filtration of acid buttermilk cheese whey with membranes of different molecular size cut-off values and membrane material (PES = polyethersulfone; CA = cellulose acetate), and with or without thermocalcic aggregation (TA)

Experiment	Retention coefficient (% $\pm$ SD)					
	DM	Ash	Protein	Reducing sugars	Lipids	Polar lipids
1) 30-kDa PES	37.9 $\pm$ 0.5	5.6 $\pm$ 0.1	82.5 $\pm$ 0.0	7.2 $\pm$ 0.0	99.9 $\pm$ 3.0	98.8 $\pm$ 3.3
2) 0.1- μ m PES	11.3 $\pm$ 0.3	0.3 $\pm$ 0.0	37.2 $\pm$ 0.4	0.3 $\pm$ 0.0	64.6 $\pm$ 2.2	35.8 $\pm$ 3.4
3) 0.15- μ m CA	13.3 $\pm$ 0.2	0.5 $\pm$ 0.0	37.5 $\pm$ 0.0	4.1 $\pm$ 0.1	73.0 $\pm$ 4.6	41.9 $\pm$ 2.4
4) 0.2- μ m CA	7.4 $\pm$ 0.1	0.3 $\pm$ 0.0	29.3 $\pm$ 0.0	1.6 $\pm$ 0.0	49.7 $\pm$ 1.4	12.2 $\pm$ 1.2
5) 0.2- μ m PES	8.9 $\pm$ 0.2	2.0 $\pm$ 0.0	34.7 $\pm$ 1.0	2.6 $\pm$ 0.1	56.9 $\pm$ 3.5	20.0 $\pm$ 1.3
6) 0.45- μ m CA	9.9 $\pm$ 0.0	1.7 $\pm$ 0.0	29.7 $\pm$ 0.5	4.7 $\pm$ 0.0	54.2 $\pm$ 2.1	14.5 $\pm$ 1.6
7) 0.15- μ m CA + TA	45.8 $\pm$ 0.6	61.0 $\pm$ 3.3	73.4 $\pm$ 3.3	9.6 $\pm$ 0.1	96.4 $\pm$ 12.5	98.4 $\pm$ 5.8
8) 0.45- μ m CA + TA	45.1 $\pm$ 3.7	58.5 $\pm$ 1.2	75.9 $\pm$ 2.6	6.6 $\pm$ 0.3	93.4 $\pm$ 7.3	93.9 $\pm$ 1.2

thermocalcic aggregation pretreatment (experiments 7 and 8), this ratio differs significantly from the whey and the other experiments. For the 30 kDa PES experiment, this seems logical, because at a pH of 3.5 to 5.2, β -LG primarily forms an octamer of 18.3 kDa units, although smaller aggregates can occur (Walstra et al., 2006). This can be clearly seen on Figure 1, lane 1: α -LA is capable of passing through the membrane to a certain extent, whereas all other proteins remain in the retentate. However, a similar phenomenon occurs upon thermocalcic aggregation pretreatment. In both TA experiments, protein is highly retained (<73%) by the membrane. From the SDS-PAGE analysis (lanes 7 and 8), it can be clearly noticed that for these 2 experiments, next to MFGM proteins, whey proteins are retained as well. Furthermore, β -LG is retained more than α -LA. Apparently, β -LG is incorporated in the aggregates to a certain extent by the thermocalcic aggregation process. From Figure 1 (lanes 1, 7, and 8), it can be further observed that, together with the other MFGM proteins, the proteose peptone 3 components completely remain in the retentate, and thus at least make part of the formed aggregates.

From Table 3, it can be noticed that ash was hardly retained by the membrane, except for the experiments with thermocalcic aggregation pretreatment (experiments 7 and 8). Here, retention is exceeding 60%, which means that most of the added Ca is involved in the formation of the aggregates. As such, the ash content of the permeates (both 0.70 ± 0.10 g/100 g) was only slightly increased compared with the whey (0.62 ± 0.02 g/100 g). Reducing sugars are, as expected, only slightly retained (0.3 to 9.6%) in all experiments. For all experiments, the retention of total lipids is higher than (or equals) the retention of PL. Particularly, for the 0.45- μ m CA membrane experiment (experiment 6), 54% of the lipids were retained, whereas only 14.5% of the PL were.

The 0.15- μ m CA membrane (experiment 3) was chosen for further MFGM purification studies. This mem-

brane combines a high permeate flux with a low protein retention and does not alter the original β -LG/ α -LA ratio. All other membranes had major drawbacks. The 30-kDa PES membrane and all filtrations preceded by a TA pretreatment exhibited a low permeate flux and a very high protein retention, which impairs further MFGM purification. Although the 0.2- and 0.45- μ m membranes (experiments 4, 5, and 6) showed a high permeate flux, their retention of PL and MFGM proteins was unacceptably low, which would lead to unacceptable losses. The 0.15- μ m CA membrane scored moderately on PL retention. Adjustment of pH and temperature has a severe influence on filtration characteristics and retention of different components and will as such have an influence on MFGM fragment retention (Cheryan, 1998).

Effect of Temperature and pH on Filtration

The effect of temperature and pH on the filtration of acid buttermilk cheese whey was determined by means of an experimental design (response surface full central composite orthogonal design). Retention and fouling coefficients were taken as response variables. The design was constrained between 15 and 50°C and pH 4 to 7.5. Thirteen design points (= filtration experiments) were defined, of which 4 were factorial points, 4 axial points, and 5 center points. The latter points determined the pure (experimental) error. All filtration experiments were carried out with 10 L of whey until a VCR of 4 was reached (2.5 L of retentate, 7.5 L of permeate), this at a TMP of 0.1 MPa, and a feed velocity over the membrane of 2.14 m/s. Linear, quadratic, and cubic statistical models were fit to the experimental data (fouling coefficient and retention factors). Quadratic models, typical for response surface, were found highly significant, and were further evaluated. Nonsignificant model coefficients were eliminated by a stepwise addition algorithm, resulting in a reduced quadratic model. The hierarchy of the model was, however, maintained,

Table 4. Statistical parameters of reduced quadratic models in function of temperature and pH of acid buttermilk cheese whey filtration with a 0.15- μ m cellulose acetate membrane

ANOVA term	Fouling coefficient	Retention coefficient		
		Protein	Lipid	Polar lipid
Sum of squares				
Model	0.095	2,963.59	466.54	4,783.19
Residual	0.00645	149.22	75.19	86.27
Lack of fit	0.00540	106.42	17.38	7.94
Pure error	0.00105	42.81	57.82	78.33
Corrected total	0.10	3,112.82	541.73	4,869.47
Predicted residual	0.016	573.62	213.91	159.17
Significance (<i>P</i> -value)				
Model	<0.0001	<0.0001	0.0065	<0.0001
Lack of fit	0.13	0.20	0.76	0.98
Correlation coefficient				
R ²	0.94	0.95	0.86	0.98
Predicted R ²	0.84	0.82	0.61	0.97

meaning that if a quadratic term is found significant, the linear term is also included, regardless of its significance. In Table 4, the ANOVA sum of squares, fitted regression coefficients, and *t*-tests are given for the reduced quadratic models of the fouling coefficient and retention coefficients of protein, lipids, and PL. The mentioned predicted residual (sum of squares) was calculated by recalculating the model coefficients using the data points minus one. This new model was then used to estimate the value for the nonincluded point. This was done for each point, and the difference between each predicted and actual value was squared and summed. The predicted R²-value is based on this predicted residual sum of squares and gives information about how well the model fits each point in the design and if the model is prone to small variations of one of the data points. The value should be close to 1.

The (reduced) quadratic model for the fouling and retention coefficients were all found to be highly significant (Table 4). Lack of fit tests, where the pure error is compared with the rest of the residual error (= lack of fit error) were found insignificant for all variables. No significant outliers (raw data vs. predicted data)

were observed, and both correlation coefficients (normal and predicted) were close to 1 for all variables. Out of all these parameters, it can be strongly concluded that the reduced quadratic models are adequate for the estimation of the fouling and retention coefficients, within the temperature and pH limits of the design. In Table 5, the significance of each model coefficient and final equation terms are given. The latter can be used for response prediction. All considered responses were highly dependent on the pH. The effect of temperature was less pronounced; only the quadratic term was found significant. The fouling coefficient was even found to be completely independent of the temperature. Except for the retention coefficient of lipids, no significant interaction terms were found, which points out that the effect of pH is independent of temperature and vice-versa.

In Figures 3 and 4, the contour plots of the retention coefficient of PL and proteins, respectively, are given. Both plots show a similar pattern. Upon increase of the pH, the retention slightly decreases with a minimum around pH 4.7 and then rises sharply. Upon increase of the temperature, the retention decreases, followed

Table 5. Significance of reduced quadratic model coefficients and final equation terms for prediction of the fouling coefficient and retention factors (RF) upon filtration of acid buttermilk cheese whey with a 0.15- μ m cellulose acetate membrane at pH of 4 to 7.5 and temperature of 15 to 50°C¹

Model coefficient	<i>P</i> -value				Final equation term			
	Fouling component	RF protein	RF lipids	RF polar lipids	Fouling component	RF protein	RF lipids	RF polar lipids
Intercept					0.46	144.76	93.52	202.13
Temperature	NS	0.4961	0.3027	0.5470	0	-2.86	-0.63	-3.61
pH	<0.0001	<0.0001	0.0121	<0.0001	0.22	-32.39	-2.09	-40.95
Temperature $\times$ pH	NS	NS	0.0496	NS	0	0	-0.13	0
Temperature ²	NS	<0.0001	0.0018	<0.0001	0	0.043	0.020	0.056
pH ²	<0.0001	0.0002	0.1136	<0.0001	-0.024	3.42	0.73	4.32

¹Some nonsignificant (NS) terms were included in the model in order to respect the model hierarchy.

Figure 3. Polar lipid retention coefficient as a function of temperature and pH upon filtration of acid buttermilk cheese whey with a 0.15- μ m cellulose acetate membrane.

Figure 4. Protein retention coefficient as a function of temperature and pH upon filtration of acid buttermilk cheese whey with a 0.15- μ m cellulose acetate membrane.

Figure 5. Lipid retention coefficient as a function of temperature and pH upon filtration of acid buttermilk cheese whey with a 0.15- μ m cellulose acetate membrane.

by a minimum around 30 to 35°C and then increases again. In Figure 5, the contour plot of lipid retention is given. Here, the effect of pH and temperature on lipid retention is less pronounced. A significant interaction is observed: at 50°C, almost no pH effect is observed, whereas at 15°C, there is an increase of lipid retention upon pH increase). In Figure 6, the fouling coefficient is given as a function of the pH. From Table 5, it can be noticed that the temperature term is insignificant and is therefore omitted as a factor in the fouling coefficient model. Fouling was highest at pH 4.6 to 4.7 and decreased sharply upon increase of the pH. In Figure 7, permeate fluxes are given for different time-temperature combinations. Here, as expected, the temperature showed the highest influence. At 15°C, the flux was very low due to the higher viscosity. Here, no pH dependency was observed. At 50°C and pH 7.5, the whey permeate flux was significantly higher than at pH 4.

Kanno and Kim (1990) found the isoelectric point of the whole MFGM fragment to be around 4.8. This is the pH at which retention of PL and proteins was found to be minimal and fouling maximal. Above a pH of 4.8, these fragments are progressively negatively charged. Following the manufacturer, the CA filtration membranes are also negatively charged. As such, at a pH > 4.8, repulsion between MFGM particles and the filtration membrane occurs. As a result, at pH > 4.8 the retention will be much higher and fouling and polariza-

Figure 6. Fouling coefficient as a function of pH upon filtration of acid buttermilk cheese whey with a 0.15- μm cellulose acetate membrane. The temperature term was found insignificant.

Figure 7. Permeate flux during the filtration of acid buttermilk cheese whey using a 0.15- μm cellulose acetate (CA) membrane at different temperature ($^{\circ}\text{C}$)-pH combinations. ∇ (50, 7.5); $\blacksquare$ (32.5, 5.75); $\blacktriangledown$ (50, 4); $\bullet$ (15, 7.5); $\circ$ (15, 4). The transmembrane pressure was kept at 0.1 MPa and the feed velocity over the membrane at 2.14 m/s.

tion concentration much lower than at pH values < 4.8 , the latter explaining the pH dependency of the whey permeate flux. Free Ca^{2+} ions, present in acid whey, can possibly interact with the negatively charged MFGM fragments, allowing aggregates to be formed, which in turn will be more retained by the filtration membrane. The effect of temperature is most likely a combined effect of 2 phenomena. First, PL show a broad phase transition between 10 and 25°C (Dufour et al., 1999). As such, the fluidity of the MFGM fragments will increase and its rigidity decrease, which will decrease its retention upon filtration. Second, between 42.5 and 57.5°C , whey proteins start to unfold and their solubility decreases (Pelegrine and Gasparetto, 2005). Hydrophobic groups are exposed, making interactions between MFGM fragments and whey proteins possible. Consequently, larger structures are formed, which will be retained by the membrane.

A pH of 7.5 and a temperature of 50°C were chosen as optimal conditions for further MFGM purification by MF because a high PL retention with a high permeate flux and a low fouling coefficient are combined. Moreover, at 50°C , the lipid retention is lower than at 15°C , which is also favorable because it leads to a higher PL/lipid ratio. As such, retentions of $98 \pm 8\%$, $90 \pm 8\%$, and $59 \pm 10\%$ for PL, total lipids, and proteins, respectively, and a FC of 0.76 ± 0.06 were predicted ($\pm 95\%$ prediction interval) by the reduced quadratic models given in Table 5.

Diafiltration of Acid Buttermilk Cheese Whey

Ten liters of a new batch of acid buttermilk cheese whey was microfiltered at 50°C and at a pH of 7.5 until a VCR of 4 was reached (2.5 L of retentate, 7.5 L of permeate; diafiltration step 1). The retentate was then diluted with distilled water to its original volume (10 L). The pH was adjusted to 7.5 and microfiltered again to VCR 4 (diafiltration step 2). This was repeated another time (diafiltration step 3). All filtration experiments were performed in duplicate with a CA membrane with a pore size of 0.15 μm , at a TMP of 0.1 MPa and a feed velocity over the membrane of 2.14 m/s. The objective of a diafiltration was to wash out all membrane-permeable components to increase the MFGM components on DM. From Tables 6 and 7, it is clear that lactose, the main whey component, was almost completely washed out after 3 diafiltration steps, as well as about 75% of the ash and proteins. In contrast, roughly 65% of the lipids were retained. All permeates were completely transparent ($\lambda_{590} = 0$). From the SDS-PAGE in Figure 8, MFGM and whey proteins can be clearly observed in the retentates, whereas in the permeates, no MFGM proteins are present. Clearly, whey

Table 6. Composition (g/100 g $\pm$ SD) of retentates and permeates of buttermilk acid cheese whey after diafiltration steps 1, 2, and 3 at 50°C and pH 7.5

Item	DM	Ash	Protein	Reducing sugars	Lipids	Polar lipids	Polar lipids/DM
	<i>g/100 g</i>						
Whey	5.66 $\pm$ 0.01	0.65 $\pm$ 0.02	0.47 $\pm$ 0.01	3.37 $\pm$ 0.12	0.47 $\pm$ 0.02	0.092 $\pm$ 0.002	1.63 $\pm$ 0.04
Retentate							
Step 1	7.72 $\pm$ 0.29 ^a	1.40 $\pm$ 0.01 ^a	0.94 $\pm$ 0.09 ^a	3.58 $\pm$ 0.09 ^a	1.34 $\pm$ 0.02 ^a	0.309 $\pm$ 0.006 ^a	4.00 $\pm$ 0.17 ^a
Step 2	3.79 $\pm$ 0.52 ^b	0.96 $\pm$ 0.19 ^b	0.66 $\pm$ 0.09 ^b	0.92 $\pm$ 0.01 ^b	1.27 $\pm$ 0.03 ^a	0.169 $\pm$ 0.010 ^b	4.47 $\pm$ 0.67 ^a
Step 3	2.51 $\pm$ 0.28 ^c	0.63 $\pm$ 0.08 ^b	0.55 $\pm$ 0.07 ^b	0.34 $\pm$ 0.02 ^c	1.26 $\pm$ 0.03 ^a	0.171 $\pm$ 0.004 ^b	6.79 $\pm$ 0.78 ^a
Permeate							
Step 1	5.05 $\pm$ 0.08 ^a	0.42 $\pm$ 0.01 ^a	0.30 $\pm$ 0.01 ^a	3.24 $\pm$ 0.40 ^a	0.29 $\pm$ 0.01 ^a	Trace	
Step 2	1.23 $\pm$ 0.20 ^b	0.18 $\pm$ 0.01 ^b	0.10 $\pm$ 0.01 ^b	0.74 $\pm$ 0.32 ^b	0.08 $\pm$ 0.01 ^b	Trace	
Step 3	0.37 $\pm$ 0.13 ^c	0.11 $\pm$ 0.01 ^c	0.05 $\pm$ 0.01 ^c	0.31 $\pm$ 0.02 ^b	0.12 $\pm$ 0.06 ^{ab}	Trace	

^{a-c}Means of retentates and permeates within a column not sharing a common superscript differ at the 0.05 significance level.

proteins are washed out (lane 5 to 7). The presence of whey proteins in retentate 3 could indicate that whey proteins are involved in the aggregate formation. Only traces of PL were found in the permeates. Assuming a PL recovery of 80%, a theoretical PL/DM content of about 11% should be obtained after 3 diafiltration steps. The actual value was however much lower. Compared with the original whey, the PL/DM content was only increased about 4 times after 3 diafiltration steps. In the first step, the PL/DM content and the recovery were comparable with those from the former experiments. However, in the second diafiltration step, about 40% of total PL remained on the filtration membrane. During the third step, no further losses occurred. The overall PL loss was about 55%. Most likely, the addition of distilled water in combination with the continuous pumping lead to the disintegration of the Ca²⁺-MFGM aggregates, which could subsequently enter the membrane pores.

CONCLUSIONS

Membrane filtration seems the appropriate technique for MFGM purification from dairy by-products. Because no casein micelles are present in whey that could block the filtration membranes upon processing, whey is an interesting source for further MFGM purification. The choice of membrane material and pore size are, however, have a great influence on filtration characteristics and MFGM retention. Negatively charged

membranes exhibit a higher flux and less fouling than apolar membrane types. The membrane pore size should be at least less than 0.15 μ m to avoid excessive MFGM losses. Thermocalcic aggregation is appropriate for whey clarification purposes, but in combination with MF, the low permeate flux and high ash and protein retentions (also β -LG) make it less suitable as a MFGM purification technique. Especially the pH had a profound effect on MFGM retention. By adjusting the pH to 7.5, the PL retention was increased to 98%. The SDS-PAGE revealed that whey proteins and the protease peptone 3 fraction, whether or not they are true MFGM proteins, are at least involved in the aggregate formation. Upon diafiltration, major losses were observed during the second step. Possibly, higher feed velocities could lower this, however, implying higher energy costs. Alternatively, smaller membrane pore sizes could be chosen, improving the recovery and lowering fouling phenomena but inherently lowering the permeate flux. These adaptations, together with the use of other whey sources like butter serum, the richest dairy source of PL, could lead to industrially feasible purification of MFGM fragments, which could be marketed as an additive with nutritional and technological enhanced properties.

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Table 7. Percentage recovery ($\pm$ SD) in the retentate compared to the original content in acid buttermilk cheese whey after diafiltration step 1, 2, and 3 at 50°C and pH 7.5

Diafiltration	DM	Ash	Protein	Reducing sugars	Lipids	Polar lipids
Step 1	34.1 $\pm$ 1.3	54.3 $\pm$ 1.9	49.7 $\pm$ 5.0	26.5 $\pm$ 1.2	70.5 $\pm$ 3.0	83.8 $\pm$ 2.6
Step 2	16.2 $\pm$ 2.2	36.3 $\pm$ 7.1	33.7 $\pm$ 4.7	6.5 $\pm$ 0.2	65.9 $\pm$ 3.1	45.5 $\pm$ 3.0
Step 3	10.3 $\pm$ 1.2	23.1 $\pm$ 3.0	27.4 $\pm$ 3.2	2.3 $\pm$ 0.2	64.4 $\pm$ 3.0	44.6 $\pm$ 1.5

Figure 8. The SDS-PAGE of butterserum whey retentates and permeates after diafiltration at 50°C and at a pH of 7.5. Lane 1: molecular weight marker; lane 2 to 4: diafiltration retentate step 1 to 3; lane 5 to 7: diafiltration permeate step 1 to 3; and lane 8: buttermilk whey. The vertical streaking in lane 4 was caused by the presence of polar lipids. XO = xanthin oxidase/dehydrogenase; CD36 = cluster of differentiation 36; IGH = immunoglobulin heavy chain; ADPH = adipophilin; PAS 6/7 = periodic acid Schiff 6/7; PP3 = protease 3 fraction 1 and 2.

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