



Quantification of MFGM proteins in buttermilk and butter serum by means of a stain free SDS-PAGE method



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ARTICLE INFO

Article history:

Received 9 January 2016

Received in revised form 15 March 2016

Accepted 8 April 2016

Available online 11 April 2016

Keywords:

MFGM proteins

Buttermilk

Butter serum

SDS-PAGE

Stain free technology

Tryptophan

Protein quantification

ABSTRACT

In comparison to skim milk and whey, buttermilk and butter serum contain high amounts of functional milk fat globule membrane (MFGM) proteins. Quantification of MFGM proteins is difficult because no analytical standard material is available. By means of conventional SDS-PAGE with subsequent staining, MFGM proteins are analytically separated. However, due to various protein structures (e.g., glycosylation) the affinity to the dye is inconsistent. A direct correlation between measured band intensity and protein concentration and, therefore, absolute protein quantification is not possible. The purpose of this study was to apply a novel stain free SDS-PAGE technique that relies on measuring UV light fluorescence of tryptophan in order to quantify MFGM proteins. Thus, proteins can be quantified without the need for analytical standards, provided that the relative tryptophan content relative to the molecular weight is known. In this study, buttermilk and butter serum obtained from raw cream were examined and the three most abundant membrane proteins were absolutely quantified. Higher amounts of peripheral MFGM proteins were determined in buttermilk (0.9 g/L) and butter serum (2.7 g/L), in comparison to integral MFGM proteins (0.3–0.5 g/L). Conclusions were drawn regarding the absolute protein amount of MFGM material surrounding native milk fat globules.

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1. Introduction

Fat globules in milk are surrounded by a multi-layered membrane (MFGM). It is well described that the MFGM is developed during fat synthesis and secretion of fat droplets from mammary epithelial cells and originates from the plasma membrane (Anderson and Cawston, 1975; Brown, 2001; Keenan and Mather, 2006). However, the detailed composition of MFGM material is controversially discussed and is not yet completely understood. For instance, the protein content of MFGM structures is reported between 25 and 70% of the MFGM dry matter (Danthine et al., 2000; Walstra et al., 2006; Singh, 2006). These extreme deviations can possibly be explained by different processing and isolation methods that were used in several studies, as reviewed by Holzmüller and Kulozik (2016b). Buttermilk (BM) and butter serum (BS), by-products of butter production and manufacturing of anhydrous milk fat respectively, contain high amounts of MFGM proteins in comparison to skim milk or whey (Corredig and Dalgleish, 1997; Rombaut et al., 2006a). These proteins

are supposed to have bioactive properties like antibacterial activity (Hancock et al., 2002) and cancer-inhibiting effects (Spitsberg and Gorewit, 1997) and are also associated with autoimmune conditions, such as multiple sclerosis (Guggenmos et al., 2004) and autism (Vojdani et al., 2002). Therefore, the interest in isolation of MFGM material and its proteins has increased significantly in recent years. However, quantitative analysis of single MFGM proteins is required in order to evaluate at which quantities certain effects are achieved.

In general, absolute protein quantification is only possible if analytical standard material is available, which is not the case for MFGM proteins (Rombaut et al., 2006a). In previous research studies, MFGM proteins were analysed by means of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent staining of the gels with Coomassie Brilliant Blue (CBB). For instance, Britten et al. (2008) obtained that MFGM proteins represent 19% of the total protein amount in buttermilk based on staining intensities. In a few studies, absolute quantification of MFGM proteins was allegedly conducted based on staining intensities (Ye et al., 2004a, 2004b). However, these values may not represent the actual protein content because staining behaviour of proteins can vary extremely. Proteins rich in basic amino acids have a higher degree of dye binding (Tal et al., 1985),

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whereas carbohydrate moieties of glycoproteins hinder the binding of CBB and lead to poor staining (Osset et al., 1989; Moller et al., 1993).

The purpose of this study was to apply a stain-free SDS-PAGE technology without the use of any dye, in order to quantify MFGM proteins, a technology which does not require analytical standards. Protein visualisation by means of stain-free SDS-PAGE is based on a UV-light-induced chemical reaction between tryptophan (Trp) residues of proteins and trichloroethanol (TCE) that is incorporated in the SDS-PAGE gels (Ladner et al., 2004). Acylated Trp residues form, which are fluorescent under UV light (Edwards et al., 2002; Ladner et al., 2014). Holzmüller and Kulozik (2016a) have shown that there is a strict correlation between the fluorescence intensity (FI) of electrophoretically separated proteins and their mass of Trp (m_{Trp}) for proteins with a relative Trp content (Trp/protein) in the range of 0.56–5.25% relative to their molecular weight (MW). The method was validated by quantifying pure β -lactoglobulin (β -LG) and β -casein samples without the need for analytical standard material.

The aim of this study was to quantify absolute values for MFGM proteins by means of this stain-free SDS-PAGE method. BM and BS samples were produced from raw cream and butter, respectively. Thus, thermal effects on proteins and losses of MFGM material due to isolation processes like cream washing (Holzmüller and Kulozik, 2016b), undesired with regard to the purpose of this study, were avoided. The most abundant MFGM proteins xanthine oxidase/dehydrogenase (XO/XDH), butyrophilin (BTN) and periodic acid Schiff 6/7 (PAS 6/7) were analysed in all samples. The specific protein amounts of the different phases (BM and BS) were compared against each other and a total MFGM protein concentration in native milk fat globules was concluded.

In addition, the amount of total caseins and whey proteins (β -IG and α -lactalbumin (α -LA)) in BM and BS were determined by high-performance liquid chromatography (HPLC) analysis.

2. Material and methods

2.1. Raw material and sample preparation

Raw cream (32% fat) was collected directly from a local dairy (Molkerei Weißenstephan, Freising, Germany) after a separation process at 55 °C to avoid thermally-induced interactions between skim milk proteins and the MFGM. The cream was cooled rapidly to 4 °C to maintain microbial stability and processed during the next two days.

In the first step of sample preparation, the raw cream was batch churned in a Thermomix (Vorwerk, Wuppertal, Germany) at maximum rotation speed for approximately 3 min until phase inversion occurred. Overall, 3.5 L of cream were churned and 1.7 kg of butter were obtained in the form of butter grains. The aqueous phase, the buttermilk (BM₁), was filtered through a sieve (0.71 μm) and collected for further analysis. In the second step, the butter grains passed through a squeeze section, where the remaining buttermilk (BM₂) was removed. Then, the finished butter was separated into butter oil and BS according to Rombaut et al. (2006b). The butter was melted at 65 °C and centrifuged at 2400 g for 5 min. After cooling the hardened butter oil was removed and the liquid BS was collected.

2.2. Chemical analysis

2.2.1. Determination of total solids

Total solids (TS) of raw cream, BM₁, BM₂, butter and BS were determined by gravimetric difference. A microwave oven with integrated analytical scale (SMART Turbo, CEM, Kamp-Lintfort, Germany) was used. Approx. 2 g of the sample were applied to a

special adsorbent pad (CEM, Kamp-Lintfort, Germany) and dried at 105 °C until constant weight ($\Delta m < 0.2 \text{ g/2 s}$) was reached.

2.2.2. Determination of total protein content

The total protein content of raw cream, BM₁, BM₂, butter and BS was determined by means of the Dumas method using the nitrogen gas analyser system vario Max cube (Elementar Analysensysteme GmbH, Hanau, Germany). The protein concentration was calculated from the total nitrogen content using a factor of 6.38 (Jones, 1941).

2.2.3. Gel electrophoresis

2.2.3.1. Sample preparation, conditions and analysis. A stain-free SDS-PAGE method was used to analyse and quantify the MFGM proteins XO/XDH, BTN and PAS 6/7 in BM₁, BM₂ and BS. All equipment material was obtained from Bio-Rad Laboratories GmbH (Munich, Germany).

A Mini-PROTEAN Tetra system and Mini-PROTEAN[®]-TGX stain free[™] gels with a polyacrylamide gradient of 4–20% were used for all SDS-PAGE analyses. Prior to application, all samples were completely diluted with MilliQ water (1:10 (v/v)), in order to prevent overloading of the gels. The diluted samples were mixed with reducing buffer (1:1 (v/v)) consisting of 5 mL Tris-HCl, 10 mL glycerol, 8 mL SDS (10% (w/v)), 0.8 mL bromophenol blue (0.5% (w/v)) and 5 g L⁻¹ dithiothreitol. All chemicals were purchased from Sigma Aldrich (St. Louis, MO). Samples (10 μL) were loaded on the gel in separate wells. In order to determine the MW (kDa) of the proteins; 5 μL of Pprecision Plus Protein[™] standard were added on a separate lane on each gel. Samples were electrophoretically separated for approximately 30 min at 250 V, 65 mA/gel and 35 W. For protein visualisation, the gels were placed on a UV transilluminator Gel Doc[™] EZ imager and irradiated with UV light (302 nm) for 3 min.

The principle of the protein visualisation is based on a method of Ladner et al. (2004). TCE, which is incorporated in the Mini-PROTEAN[®]-TGX Stain-Free[™] gels, reacts under UV light with the Trp residues in proteins and initiates fluorescence. Photographs of each gel were taken by a Lumenera Lu130 camera under UV light. Afterwards, the pictures of the gels were analysed using Image Lab[™] software version 4.1. A chromatogram was obtained for each lane, representing the FI as a function of the relative front. Regarding the corresponding peak area, the FI was calculated for each protein band.

2.2.3.2. Protein quantification. Stain-free SDS-PAGE (Freeby et al., 2013) was applied without the need for analytical standards. All equipment was provided from Bio-Rad Laboratories GmbH (Munich, Germany) MFGM proteins (XO/XDH, BTN and PAS 6/7) in BM and BS were absolutely quantified based on the method described by Holzmüller and Kulozik (2016a). Here, a basic requirement is the correlation between Trp amount (m_{Trp}) of proteins and the corresponding FI. The higher the relative Trp content is (Trp/protein), the higher the determined FI. Trp/protein is dependent on the number of Trp residues per molecule ($N_{\text{Trp/protein}}$) and the MW_{protein} and is defined as follows:

$$\text{Trp/protein}[\%] = N_{\text{Trp/protein}} \times \frac{MW_{\text{Trp residue}}}{MW_{\text{protein}}} \times 100 \quad (1)$$

$$MW_{\text{Trp residue}} = MW_{\text{Trp}} - MW_{\text{H}_2\text{O}} = 186.21 \text{ g/mol}$$

A calibration curve consisting of five test proteins varying in Trp/protein (0.56–5.25%) was created, for which the protein concentration and, thus, m_{Trp} was known. In this study,

phosphorylase B (PB) from rabbit muscle, ovalbumin (OV) from chicken egg white, trypsin inhibitor (TI) from *Glycine max* (soybean), bovine serum albumin (BSA) and α -LA from bovine milk were used as test proteins (see Table 1). The five proteins were mixed together and the solution was diluted by factor two and four. These three test solutions (C_1 , C_2 , C_3) were separated and analysed as described above. For each protein band the FI was plotted against m_{Trp} and linear regression ($y(x) = y_0 + a \times x$) was conducted.

In order to quantify MFGM proteins in BM and BS the respective FI was determined and m_{Trp} was calculated as follows:

$$m_{\text{Trp}}/\text{band} = \frac{\text{FI} - y_0}{\text{slope } a \text{ of calibration curve}} \quad (2)$$

Final protein concentrations were calculated as follows:

$$C_{\text{protein}} = \frac{m_{\text{protein}}/\text{band}}{\text{DF} \times V_{\text{protein solution}}/\text{lane}} = \frac{((m_{\text{Trp}}/\text{band})/(\text{Trp}/\text{protein}[\%])) \times 100}{\text{DF} \times V_{\text{protein solution}}/\text{lane}} \quad (3)$$

DF: dilution factor; V: volume.

The MW, $N_{\text{Trp/protein}}$ and Trp/protein for XO/XDH, BTN and PAS 6/7 are summarised in Table 1.

2.2.4. HPLC analysis

The amount of caseins and whey proteins in BM₁, BM₂ and BS was assessed by RP-HPLC based on the methods of Bobe et al. (1998). One volume of sample was mixed with four volumes of guanidine buffer. The buffer was prepared by adding 143.295 g guanidine HCl (6 M), 1.579 g trisodium citrate (5.37 mM) and 0.755 g DTT (19.5 M). This mixture was made up to 250 mL with a BisTris buffer (0.1 M, pH 6.8). These samples were left for 30 min at room temperature. Turbid samples were filtered (0.45 μm ; Chromafil RC-45/25 Macherey-Nagel, Düren, Germany) and 20 μL of each sample were injected into the HPLC system.

In this study, an Agilent 1100 Series chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a binary pump, autosampler and UV detector was used. Separation of the proteins was performed on a Zorbax 300 SB C18 column with a silica-based packing (3.5 μm , 300 Å 150 $\times$ 4.6 mm I.D.; Agilent Technologies). Eluent A consisted of 10% acetonitrile (Sigma Aldrich, Steinheim,

Table 1

Molecular weight (MW), number of Trp residues per particle ($N_{\text{Trp/protein}}$) and relative Trp content (Trp/protein) of all used proteins.

Protein	MW (kDa) ^a	$N_{\text{Trp/protein}}$ ^a	Trp/protein (%)
α -LA	14.2	5	5.25
PB	97.2	12	2.30
TI	20.1	2	1.85
OV	42.8	3	1.31
BSA	66.4	2	0.56
XO/XDH	146.6	11	1.40
BTN	56.5	12	3.95
PAS 6/7	45.5	10	4.09

^a Based on the information from ExPASy-ProtScale [<http://web.expasy.org>].

Germany) in HPLC-grade water, containing 1 mL/L (v/v) trifluoroacetic acid (TFA). Eluent B consisted of 90% acetonitrile in HPLC-grade water, containing 0.7 mL/L TFA (Merck, Darmstadt, Germany). The gradient elution programme was run at a constant flow rate of 1.2 mL/min and was set as follows: 0–2 min linear gradient from 27% B to 32% B; 2–10 min linear gradient from 32% B to 38% B; 10–13 min isocratic elution at 38% B; 13–16 min linear gradient from 38% B to 40% B; 16–20 min linear gradient from 40% B to 42% B; 20–22 min linear gradient from 42% B to 44% B; 22–22.5 min linear gradient from 44% B to 0% B; 22.5–25 min isocratic elution at 0% B; 25–26 min linear gradient from 0% B to 100% B; 26–28 min isocratic elution at 100% B; 28–28.5 min linear gradient from 100% B to 27% B followed by 2.5 min isocratic elution at initial conditions. The calibration was performed using pure casein and whey protein standards (Sigma Aldrich, St. Louis, MO). Peak detection was at 220 nm. The total casein amount was determined by summarising the peak areas of the four casein fractions κ -, α_{S1} -, α_{S2} - and β -casein. The amount of β -LG was determined by summarising the peak areas of the two genetic variants A and B. A chromatogram of the separated caseins and whey proteins is depicted in Fig. 1.

2.3. Statistical analyses

Each experiment was carried out in triplicate. One-way analysis of variance was carried out to determine significant differences ($p < 0.05$) using SigmaPlot for Windows (version 12.3, build 12.3.0.36; Systat Software Inc., Chicago, IL).

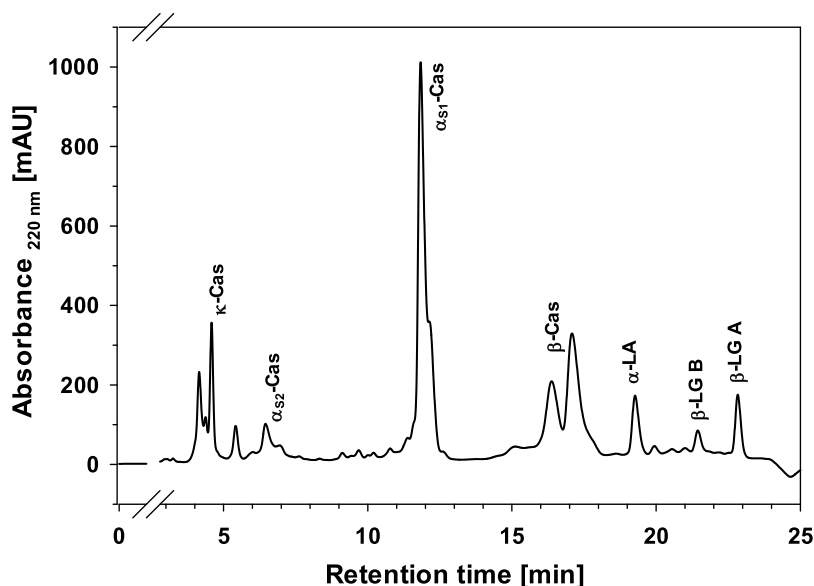


Fig. 1. Separation of caseins and whey proteins by RP-HPLC. Chromatogram of a buttermilk sample.

3. Results and discussion

3.1. Compositional characterisation of different fractions and by-products

Raw cream was processed as described above. TS and total protein content of initial, intermediate and end products are summarised in Table 2. For raw cream (32% fat) a TS of 37.27% was determined. The fat:TS ratio of 0.86 is consistent with literature values (Rombaut et al., 2005, 2006b, 2007). The protein content of the raw cream was 2.67%. The protein amount in cream depends on the fat content. At higher fat contents, the amount of skim milk proteins (casein, whey protein) decreases, whereas the amount of MFGM proteins increases. The higher the fat content the more fat globules are in the cream and, thus, a higher amount of MFGM material and incorporated membrane proteins is present (Mulder and Walstra, 1974).

In this study, raw cream was batch churned for approximately 3 min and equal amounts of butter grains and BM₁ were obtained. Butter grains are fat particle aggregates (0.1–2.0 cm) with a higher water content (~20%) compared to common butter (15–16%). Hence, butter grains were squeezed in order to remove residual water (indicated as BM₂) and to obtain a homogenised butter structure.

BM₁ shows higher values for TS (11.70%) than BM₂ (10.55%), whereas the protein content was in a similar range (BM₁: 3.39%, BM₂: 3.36%). Although BM₁ was filtered through a sieve (0.71 µm), small butter grains, which mainly consist of lipids, might remain in the aqueous phase. Thus, a higher TS content was detected that results in a lower protein/TS amount (BM₁: 28.97%; BM₂: 31.85%).

In general, gross protein composition of buttermilk is similar to skim milk apart from relatively high amounts of MFGM proteins. During the churning process MFGM material that surrounds fat globules in milk passes into the aqueous phase due to its hydrophilic properties. However, the ratio of MFGM proteins to skim milk proteins in bovine buttermilk varies across the literature between 0.23 (Britten et al., 2008) and 0.85 (Morin et al., 2007a).

BS, the aqueous phase of butter, had a higher TS content (12.05%) in comparison to BM₁ and BM₂, which could be due to the higher amount of phospho- and sphingolipids (Rombaut et al.,

Table 2

Total solids (TS), and total protein content of raw cream, buttermilk (BM₁ and BM₂), butter and butter serum (BS).

Sample	TS (%) ^a	Protein (%) ^a	Protein/TS (%) ^a
Raw cream	37.27 ± 0.91 ^a	2.67 ± 0.01 ^a	7.17 ± 0.16 ^a
BM ₁	11.70 ± 0.09 ^{b,d}	3.39 ± 0.29 ^b	29.00 ± 1.91 ^b
BM ₂	10.55 ± 0.13 ^b	3.36 ± 0.06 ^b	31.84 ± 0.55 ^c
Butter	85.13 ± 0.33 ^c	1.16 ± 0.13 ^c	1.36 ± 0.11 ^d
BS	12.05 ± 0.31 ^d	4.32 ± 0.01 ^d	35.83 ± 0.8 ^e

a,b,c,d,e Different superscripts within a column indicate significant differences ($p < 0.05$).

^a Data are means ± sample SD.

2006b; Lamothe et al., 2008). A significantly higher protein content (4.32%) and thus a higher protein/TS amount (35.85%) was also obtained in comparison to both BM samples.

Differences of individual protein concentrations in BM and BS were further analysed. These results are discussed in Sections 3.3 and 3.4.

3.2. Preparation of the calibration curve

In order to implement absolute protein quantification by means of the stain-free SDS-PAGE method a calibration curve is required. In this study a protein solution consisting of the five test proteins α-LA, PB, TI, OV and BSA was used and applied on SDS-PAGE gels at three different concentrations (C₁–C₃). In Fig. 2 such an SDS-PAGE gel is depicted. Clear and well separated protein bands were obtained after gel electrophoresis and subsequent UV irradiation. In general, the higher the Trp/protein the higher the FI that can be detected at similar protein concentrations (Ladner et al., 2004; Holzmüller and Kulozik, 2016a).

In Fig. 2 the measured FI of each protein band of C₁–C₃ was plotted against the corresponding m_{Trp}. Due to the fact that solely Trp residues in proteins react with TCE in the gels at UV irradiation, the measured FI strictly correlates with the m_{Trp} (Holzmüller and Kulozik, 2016a). Linear regression was conducted and a good correlation of $r^2 = 0.98$ was obtained up to m_{Trp} of 105.3 ng. This calibration curve was used for further quantification of MFGM proteins.

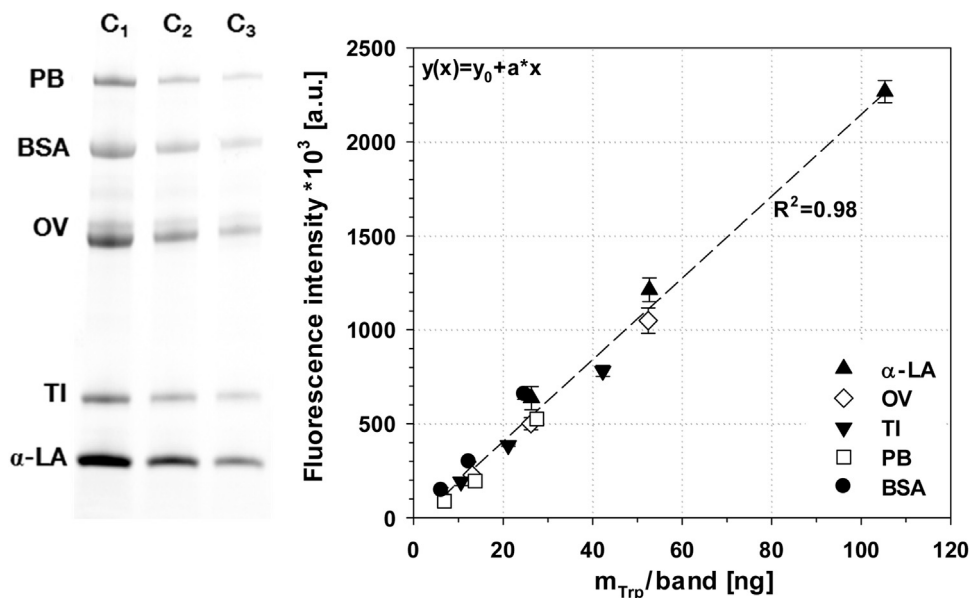


Fig. 2. SDS-PAGE pattern after UV irradiation and TCE reaction of the test protein solution (C₁–C₃) containing phosphorylase B (PB), bovine serum albumin (BSA), ovalbumin (OV), trypsin inhibitor (TI) and α-lactalbumin (α-LA) alongside a calibration curve based on measured fluorescence intensity and mass of Trp (m_{Trp}) per protein band. Error bars represent standard deviations of mean data from triplicate experiments.

3.3. Quantification of MFGM proteins in buttermilk

The quantification of MFGM proteins in BM was conducted by means of the stain free SDS-PAGE technology. BM samples were electrophoretically separated and examined with UV light as described above. SDS-PAGE pattern of BM₁, BM₂ and the protein standard (MW (kDa)) are exemplarily depicted in Fig. 3.

In addition to intensive bands of caseins and whey proteins, clear bands of the three proteins XO/XDH (136 ± 4 kDa), BTN (62 ± 2 kDa) and PAS 6/7 (46 ± 1 kDa), which occur most often in MFGM (Danthine et al., 2000), were analysed.

The measured FI of each protein band was used as an initial value in order to quantify MFGM proteins in the BM samples. The protein content was calculated according to Eqs. (2) and (3). Absolute protein concentrations (g/L product) of XO/XDH, BTN and PAS 6/7 are shown in Fig. 4. No significant differences were obtained between BM₁ and BM₂. However, the amount of PAS 6/7 (~0.85 g/L) was much higher in both BM samples in comparison to XO/XDH (~0.50 g/L) and BTN (~0.35 g/L). BM contains a total of 1.7 g/L of these three MFGM proteins, adding up to a proportion of the total BM protein of approximately 5% (see Fig. 5).

BTN is often described as the most abundant protein in bovine MFGM (about 40% of total MFGM protein content (Mather, 2000; Danthine et al., 2000; Riccio, 2004; Spitsberg, 2005)). Mondy and Keenan (1993) found an XO/XDH:BTN ratio of around 0.24 whereas the opposite was detected in our study. Here, the XO/XDH:BTN ratio was approx. 1.4 in both BM samples. In addition, our results showed that BTN made up only 21%, whereas PAS 6/7 made up 50% relative to the three main MFGM proteins. These differences between results from different researchers are most likely a result of different sample preparation methods, e.g., cream washing, which cause inconsistent losses of MFGM material, but also a result of different analytical techniques applied in other studies (Holzmüller and Kulozik, 2016b).

In relation to a fat content of 32% of the raw cream, 1.09 mg BTN/g fat, 1.56 mg XO/XDH/g fat and 2.66 mg PAS 6/7/g fat were determined in the BM samples. Ye et al. (2004b) determined 1.1 mg BTN/g fat, 0.9 mg XO/XDH/g fat and approximately 1.4 mg PAS 6/7/g fat in MFGM material isolated from early season milk. Although

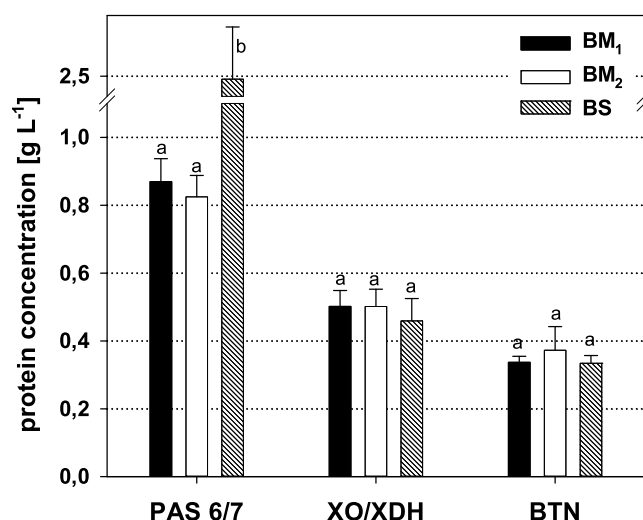


Fig. 4. Protein content of xanthine oxidase/dehydrogenase (XO/XDH), butyrophilin (BTN) and periodic acid Schiff 6/7 (PAS 6/7) in buttermilk (BM₁, BM₂) and butter serum (BS) analysed by means of stain free SDS-PAGE. Error bars represent standard deviations of mean data from triplicate experiments. Different letters (a,b) indicate significant differences ($p < 0.05$) between BM₁, BM₂ and BS.

PAS 6/7 was the most abundant MFGM protein in the work of Ye et al. (2004b), we obtained around twice as much PAS 6/7. However, in previous studies quantification of MFGM proteins was based on CBB staining. Consequently, different degree of dye binding can be expected. The carbohydrate moieties of PAS 6/7 hinder the binding of CBB (Osset et al., 1989; Moller et al., 1993) and, thus, lower protein concentrations were obtained.

Furthermore, isolated MFGM material was used in many studies. However, a specific MFGM protein loss may occur during these isolation procedures (Holzmüller et al., 2016; Le et al., 2009; Zheng et al., 2013), which was not taken into consideration in previous studies.

Thus, the amount of MFGM proteins and their quantitative distribution differs between fresh buttermilk and isolated MFGM material. For instance the amount of the peripheral membrane protein PAS 6/7 is significantly higher in fresh buttermilk, which has to be considered for further studies on the functional potential of buttermilk components and future applications of buttermilk in food systems.

3.4. Quantification of MFGM proteins in butter serum

BS, a by-product of anhydrous milk fat production, is the aqueous phase of butter and contains high amounts of MFGM proteins (see Fig. 3). Protein quantification and evaluation for BS was conducted analogously to BM in Section 3.3.

The absolute protein amount of BTN (0.33 g/L) and XO/XDH (0.46 g/L) in BS was similar as in BM₁ and BM₂ (see Fig. 4). However, the relative protein content of MFGM proteins relative to the total protein content (g/100 g total protein) decreased significantly, which is shown in Fig. 5. The proportion of the protein content in BS decreased almost 30% in comparison to BM₂ from 1.5% to 1.1% for XO/XDH and from 1.1% to 0.8% for BTN, respectively. In contrast, an extreme increase for PAS 6/7 was obtained. The content of PAS 6/7 (2.49 g/L) in BS was three times as high as for BM₁ and BM₂, which makes up 5.8% of the total BS protein and around 75% of the MFGM proteins in BS (see Figs. 4 and 5).

This was not expected in such an extent because several published MFGM models described PAS 6/7 as a peripheral and loosely bound membrane protein (Dewettinck et al., 2008; Vanderghem et al., 2011), due to the fact that PAS 6/7 can be

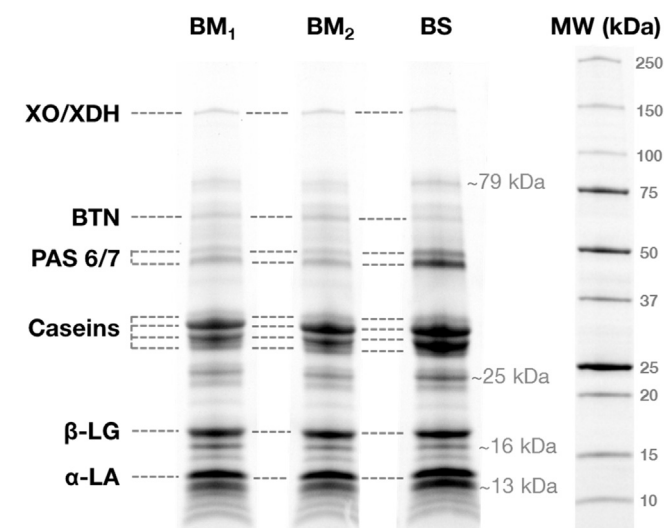


Fig. 3. SDS-PAGE pattern after UV irradiation and TCE reaction of buttermilk (BM₁, BM₂) and butter serum (BS) containing the MFGM proteins xanthine oxidase/dehydrogenase (XO/XDH), butyrophilin (BTN) and periodic acid Schiff 6/7 (PAS 6/7), the caseins and the whey proteins β-lactoglobulin (β-LG) and α-lactalbumin (α-LA). MW (kDa): Precision Plus Protein™ standard. Protein bands at 79, 25, 16 and 13 kDa were not further identified in this study.

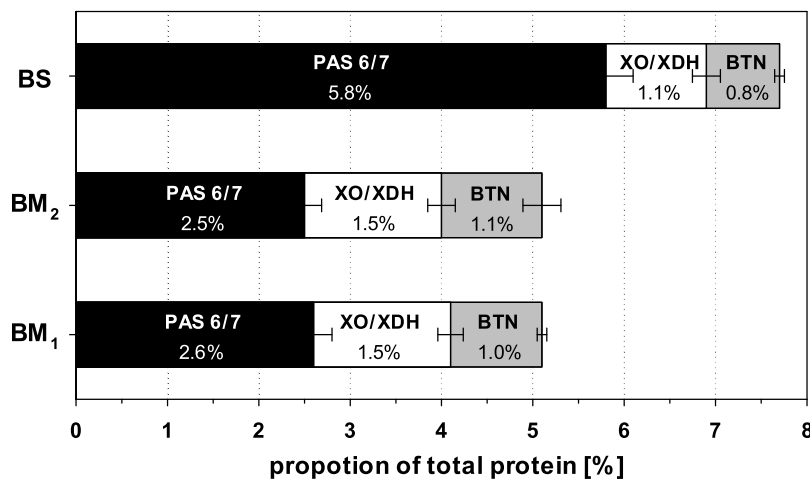


Fig. 5. Portion of xanthine oxidase/dehydrogenase (XO/XDH), butyrophilin (BTN) and periodic acid Schiff 6/7 (PAS 6/7) in relation to the total proteins in buttermilk (BM₁, BM₂) and butter serum (BS).

easily removed from fat globules, for example by cream washing (Mather et al., 1977; Shimizu et al., 1978; Nielsen et al., 1999).

Our results indicate that different amounts of MFGM proteins merge into the BS in comparison to BM during the churning process, which confirms a heterogeneous MFGM structure (Evers et al., 2008; Lopez et al., 2010). Regarding the structural ordering of lipids in the MFGM, the choline-containing phosphatidylcholine (PC) and sphingomyelin (SM) are largely located on the outside of the MFGM, while the neutral lipids phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) are concentrated on the inner membrane surface (Deeth, 1997; Keenan and Patton, 1995; Lopez et al., 2010). Higher amounts of PC and SM are in butter and BS, whereas higher amounts of PE, PS and PI were detected in BM (Rombaut et al., 2005, 2007). According to our results, it can be concluded that XO/XDH and BTN are connected to the inner (PE, PS, PI) as well as to the outer layer (PC, SM) of the MFGM, whereas PAS 6/7 is concentrated mainly at the outer membrane surface (PC, SM). Although this structural arrangement is consistent with existing MFGM models, we would like to point out that PAS 6/7 seems to be not that loosely bound to lipids in the MFGM backbone as previously assumed.

3.5. Determination of skim milk proteins in buttermilk and butter serum

Along with MFGM proteins high amounts of skim milk proteins (caseins and whey proteins) can be found in BM and BS (see Fig. 3). To complete the picture, we analysed the protein amount of total caseins, β -LG, and α -LA by means of HPLC. The data are summarised in Table 3. Caseins and whey proteins made up almost 85% of the total protein in BM and around 80% of the total protein in BS.

The casein amount was significantly higher in BS in comparison to BM₁ and BM₂ due to the higher total protein content in BS. However, the relative casein amount relative to the total protein content was not significantly different. A decrease of 20% was obtained for β -LG and α -LA comparing their relative protein content between BM and BS. Thus, whey proteins show higher solubility in BM than in BS. Despite the fact that caseins are also expected to be highly soluble, the increased casein:whey protein ratio from 4 to 5 is caused by the churning process. A partial homogenisation may occur during churning of cream, causing caseins to interact with MFGM fragments (Morin et al., 2007b) and thus, caseins are recovered in higher amounts in the BS in comparison to whey proteins.

3.6. MFGM protein composition of native fat globules

Based on the protein quantification of BM and BS (Fig. 4) and the mass portion of BM (50%) and BS (10%) obtained during cream and butter processing, the total amount of the main MFGM proteins XO/XDH, BTN and PAS 6/7 in raw cream was determined. For BM the average values of BM₁ and BM₂ were used; 1.18 g MFGM proteins/L raw cream (XO/XDH: 0.30 g/L; BTN: 0.21 g/L; PAS 6/7: 0.67 g/L) were obtained in this study. Due to the fact that raw cream was used, this protein content can be related to the membrane material surrounding native fat globules in cream. Neither cream heating/pasteurisation nor MFGM isolation procedures were used, which would in general significantly change the structure and composition of MFGM material (Houlihan et al., 1992; Ye et al., 2004b; Holzmüller et al., 2016). With regard to the fat content of raw cream (32%) 3.69 mg MFGM proteins/g fat (XO/XDH: 0.93 mg/g fat; BTN: 0.65 mg/g fat; PAS 6/7: 2.11 mg/g fat) were obtained in this study. MFGM proteins and polar lipids make up over 95% of the

Table 3

Protein concentration of total caseins, β -lactoglobulin and α -lactalbumin in BM₁, BM₂ and BS.

	Total caseins ^a		β -Lactoglobulin ^a		α -Lactalbumin ^a	
	(g/L product)	(g/100 g total protein)	(g/L product)	(g/100 g total protein)	(g/L product)	(g/100 g total protein)
BM ₁	22.94 ± 0.67 ^a	67.67 ± 1.96 ^a	4.38 ± 0.17 ^a	12.92 ± 0.49 ^a	1.16 ± 0.07 ^a	3.42 ± 0.22 ^a
BM ₂	22.58 ± 0.15 ^a	67.20 ± 0.45 ^a	4.43 ± 0.10 ^a	13.18 ± 0.30 ^a	1.18 ± 0.08 ^a	3.53 ± 0.23 ^a
BS	28.84 ± 0.05 ^b	66.76 ± 0.11 ^a	4.49 ± 0.13 ^a	10.40 ± 0.29 ^b	1.24 ± 0.16 ^a	3.09 ± 0.38 ^a

^{a,b} Different superscripts within a column indicate significant differences ($p < 0.05$).

^a Data are means ± sample SD.

membrane material (Walstra et al., 2006). Rombaut et al. (2005, 2006b) determined 3.5–4.5 mg polar lipids/g fat in cream and, thus, XO/XDH, BTN and PAS 6/7 make up 45–51% of the total MFGM.

According to our results, strong binding of PAS 6/7 to MFGM fragments rich in PC and SM can be concluded, due to the fact that during churning of cream and disruption of the MFGM at high shear forces, PAS 6/7 is released into the aqueous BM phase to a lesser extent.

4. Conclusion

Although analytical standards are not available, MFGM proteins were quantitatively analysed in this study by means of a novel stain-free SDS-PAGE technology based on their Trp amount. Pure BM and BS were able to be used without prior isolation of MFGM material as was previously required (Le et al., 2013; Patton and Huston, 1986). Hence, structural changes and loss of membrane material does not occur and measured protein concentrations can be related to protein concentrations in native fat globules.

BM₁ and BM₂ showed no significant compositional differences. However, the BS, the emulsified aqueous phase in butter, showed significant compositional differences. Higher amounts of PAS 6/7 and lower amounts of BTN and XO/XDH were obtained in BS in comparison to BM. Due to the different distribution of polar lipids in BM and BS, strong binding of PAS 6/7 to MFGM fragments rich in PC and SM can be concluded. These fragments may act as an emulsifier to stabilise the W/O emulsion in the butter phase. By melting and centrifugation of butter this emulsion is destroyed and the amphiphilic fragments pass into the BS. However, it is difficult to determine how much MFGM protein remains in the butter oil, as pure oil cannot be analysed by SDS-PAGE or HPLC.

Future studies on additional MFGM proteins (e.g., CD 36, ADPH or FAPB) and development of a 2-D stain-free SDS-PAGE, in order to analyse proteins with similar molecular weights but different isoelectric points, would further provide a better relation to physiological properties of membrane proteins surrounding intact fat globules in milk.

Acknowledgements

This research project was supported by the German Ministry of Economics and Technology (via AiF) and the FEI (Forschungskreis der Ernährungsindustrie e. V., Bonn). Project AiF 16226 N. The technical assistance of I. Hager, C. Gottwald and C. Hengst is gratefully acknowledged.

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