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Fractionation of casein micelles and minor proteins by microfiltration in diafiltration mode. Study of the transmission and yield of the immunoglobulins IgG, IgA and IgM

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ABSTRACT

There is little information concerning the fractionation by microfiltration (MF) of casein micelles and immunoglobulins plus other minor whey proteins with ceramic gradient membranes. The order of transmission was α-lactalbumin (α-La, 2.3 nm)> β-lactoglobulin (β-Lg; 4.2 nm)>IgG (10.7 nm)> lactoperoxidase (8.2 nm)> IgA (18.1 nm)> IgM (23.8 nm)> lactoferrine > blood serum albumin (7.8 nm), irrespective of the applied transmembrane pressure (0.6–3 bar) and equal to 55% > 50% > 47% > 41% >39% > 32% > 22% > 19%. Including preconcentration, it was possible to obtain 90% of the initial IgG, IgA and IgM within 85, 119, and 160 min, based on 1 m^2 of membrane area and 50 L of skim milk volume. The long-term process exposure at 50 °C did not affect α-La and IgG but β-Lg (3–5% denaturation), which, however, was selectively retained by the MF. In conclusion, MF is not only suitable for fractionation of the major whey proteins and caseins, but also for the minor and far bigger immunoglobulins.

1. Introduction

The natural function of the three immunoglobulin (Ig) classes IgG, IgA and IgM of bovine milk is to transfer the antibodies specific for certain environmental antigens from the mother to the neonate. In particular, the major function of the most abundant Ig class IgG is to bind antigens while activating the complement system and tagging pathogens for phagocytosis (Marnila & Korhonen, 2011). Dimeric secretory IgA, the major immunoglobulin class in the area of mucosal secretions, prevents mucosal infections by agglutination of microbes and neutralisation of toxins. IgM appears at the first contact with an antigen. Therefore it has a lower specificity but is also effective for the same functions of the other Ig (Hurley & Theil, 2011; Marnila & Korhonen, 2011). These functions, which can be enhanced by vaccinating cows for specific antigens (Korhonen, Marnila, & Gill, 2000; Steele, Sponseller, Schmidt, Cohen, & Tzipori, 2013), make products containing bovine antibodies a valuable source for various foods and pharmaceutical applications.

However, due to the low concentration of IgG (0.15–0.8 g L^{-1}), IgA (0.05–0.14 g L^{-1}) and IgM (0.04–1.0 g L^{-1}) in raw milk (Marnila & Korhonen, 2011), enrichment of the immunoglobulins is necessary to achieve a specific concentration that causes a biological function in humans. To increase the relative concentration of the Ig together with the other serum proteins in milk, the casein fraction, which is the major protein class in milk and which exists in micellar form, must be removed. The size difference between casein micelles (mean diameter 180 nm) and serum proteins (mean diameter 1–30 nm) allows the separation of these fractions by microfiltration (MF) (Piry et al., 2012). The main advantage of MF over other methods of removing casein, such as acid or rennet precipitation, is that MF with nominal pore sizes in the range of 0.1–0.2 μm not only allows removal of casein micelles, but also residual fat (0.2–6 μm), microorganisms (0.2–15 μm) and somatic cells (6–15 μm). Consequently, the casein free milk serum is free from cheese curd particles and virtually free of microorganisms (Gosch, Apprich, Kneifel, & Novalin, 2013; Saboyainsta & Maubois, 2000).

Existing work on milk protein fractionation by MF focused on the separation of casein micelles and the major whey proteins α-lactalbumin (α-La) and β-lactoglobulin (β-Lg) (Adams & Barbano, 2013; Kühnl et al., 2010; Steinhauer, Kühnl, & Kulozik, 2011), but there is a lack of information regarding the filtration performance of the immunoglobulins with respect to transmission and

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retention. The critical question derives from the fact that the three immunoglobulins IgG (146–163 kDa), IgA (385–430 kDa) and IgM (900 kDa) (Marnila & Korhonen, 2011) have a molecular mass 10 to 50 times larger than that of the main whey proteins (14–18 kDa). For the individual Ig classes, it was therefore unclear whether the Ig would be retained together with the casein micelles and whether, or to what extent, they would be transferred convectively with the permeate stream into the permeate.

Regarding IgG, it has been reported that it is possible to separate IgG and casein during MF of bovine colostrum (Gosch et al., 2013; Piot, Fauquant, Madec, & Maubois, 2004) and milk (Gosch et al., 2013; Heidebrecht, Toro-Sierra, & Kulozik, 2018b; Le Berre & Daufin, 1998) as well as transgenic goats' milk (Baruah & Belfort, 2004), but with inconsistent results regarding the transmission of IgG, varying from 33% (Le Berre & Daufin, 1998), 50–55% (Heidebrecht et al., 2018b) to >99% (Gosch et al., 2013). The reasons for these conflicting data are likely to be the application of different processing conditions, experimental systems and analytical techniques. Although the concentration of IgG in milk is about 8–10 times higher than that of IgA, secretory IgA is the dominant immunoglobulin class in mucosal secretions and in human breast milk and therefore has a great potential to be used for oral therapy of gastrointestinal diseases in humans (van Dissel et al., 2005). In addition, the secretory component bound to dimeric IgA protects the antibody against proteolytic degradation (Woof & Kerr, 2006), while IgG degradation during passage through the gastrointestinal tract is greater than 95% (Kelly et al., 1997). In the potential use of bovine antibodies derived from the milk of immunised cows for the treatment of gastrointestinal diseases in humans (Korhonen et al., 2000; Steele et al., 2013), it is not only important to consider at the major immunoglobulin class IgG but also at the minor fractions IgA and IgM. No scientific data have been reported on their fractionation by MF. These two minor immunoproteins are about two and five times larger in size compared with IgG, and it was unclear whether MF could be used for their fractionation.

The aim of this study was use MF to obtain whey from skim milk containing, in addition to the smaller whey proteins, the three immunoglobulin classes IgG, IgA and IgM. Although we used normal milk and not milk from immunised cows, it is expected that the immunisation will not alter the antibody properties in terms of size and charge. Hence, their filtration performance can be evaluated in a first stage with normal mature skimmed milk. First, the transmission and yield of all Ig were examined under the same conditions previously described for the fractionation of casein micelles and the major whey proteins, i.e., β-Lg and α-La (Piry et al., 2012). Due to the flexible architecture of the Y-shaped molecules, having a hinge region between the light and the heavy chains of the molecule, the hypothesis was that the Ig could behave like the smaller whey proteins in terms of transmission. However, this was more an open question than a clear expectation. Based on the same assumption of a high flexible architecture, the transmembrane pressure (TMP) was increased with the aim of pushing the proteins through the membrane and deposit layer with the overall goal of accelerating the process.

The focus of this work was on the transmission of the three milk-derived immunoglobulins IgG, IgA and IgM in milk during MF, which was operated in diafiltration mode. In this regard, we determined the required amount of diafiltration steps and filtration time to achieve a 90–95% yield of the immunoglobulins at different TMP. In addition to the yield, the quality of the obtained whey was studied with respect to the nativity of the proteins. The question was whether the processing stress in the MF unit at 50 °C for 7–8 h and the high flow velocity (approximately 6 m s^{-1}) affects the nativity of the proteins and if so, whether the MF filter membrane plus the deposit layer retains denatured and aggregated proteins.

Although not the main focus of this study, we obtained the same data as for the Ig for the other minor whey proteins blood serum albumin (BSA), lactoferrin (LF) and lactoperoxidase (LPO). The reason was that these proteins could be quantified simultaneously using the same analytic method. These data are presented in part with this publication and in part in the aligned Data in Brief publication (Heidebrecht & Kulozik, 2019).

2. Materials and methods

2.1. Microfiltration of skim milk

Raw milk was collected from a local farm and then defatted (<0.1%) as described by Heidebrecht et al. (2018a). The skimmed milk (for composition see Table 1) was subjected to the same MF unit as described elsewhere (Kühnl et al., 2010) and connected in series with an ultrafiltration (UF) unit (Fig. 1). The MF was operated in crossflow mode using ISOFLUX® (TAMI Industries, France) ceramic membranes (support and selective layer titanium dioxide) with a nominal cut-off of 0.14 μm and a membrane area of 0.35 m^2, 1178 mm length, 25 mm diameter, 23 channels and 3.5 mm equivalent hydraulic diameter per channel. The membrane unit was preheated to 50 °C with softened water for 20 min, and the first 5 L of retentate were discarded at the water to milk transition to avoid a dilution. The MF-process was initially operated at 0.6 ± 0.02 bar transmembrane pressure (TMP), 150 Pa ± 5 Pa (equivalent to 2 bar pressure drop) wall shear stress and a temperature of 50 °C ± 1 °C based on previous results (Heidebrecht et al., 2018b). The temperature was chosen for microbiological reasons, which is above growth optimum of microorganisms in milk, and because of the lower retentate viscosity associated with a higher flux compared with cold temperatures. For further experiments, the TMP was varied (1, 1.5, 2, 2.5, 3 bar), while pressure drop and temperature were identical to the previous experiments.

First, 50 L of skimmed milk was concentrated by a factor of two and the MF permeate was collected in the feed tank of a second filtration plant equipped with a 10 kDa cut-off UF SPIRA-CEL® spiral wound membrane with a membrane area of 1.8 m^2 (Microdyn-Nadir GmbH, Wiesbaden, Germany). The UF-system previously used for microfiltration experiments is described in more detail by Piry, Kühnl, Tolkach, Ripperger, and Kulozik (2008). After the preconcentration step, the UF permeate was directed to the MF feed tank and thus served as diafiltration (DF) medium for skimmed milk fractionation in a closed loop. The levels of both the MF and UF feed tanks were kept the same and constant. This was achieved by regulating the TMP for the UF system to adjust the UF flux at 50 °C to the MF flux. At least six DF steps were performed at constant filling level by transferring the permeate of the UF unit by measuring the transferred volume into the MF receiver tank with volumetric flowmeter (Promag 50H, Endress & Hauser, Weil am Rhein, Germany) and taking into account the void volume of the respective filtration plant. One DF step corresponds to one volume exchange (25 L), measured via the cumulative flux in the flowmeter UF permeate was chosen as DF medium to keep the concentration

Table 1
Protein composition of milk.

Parameter	Protein concentration (mg mL^{-1})
α-La	0.96 ± 0.07
β-Lg	4.7 ± 0.78
IgG	0.45 ± 0.066
IgA	0.065 ± 0.021
IgM	0.087 ± 0.030
Casein	35.28 ± 3.13

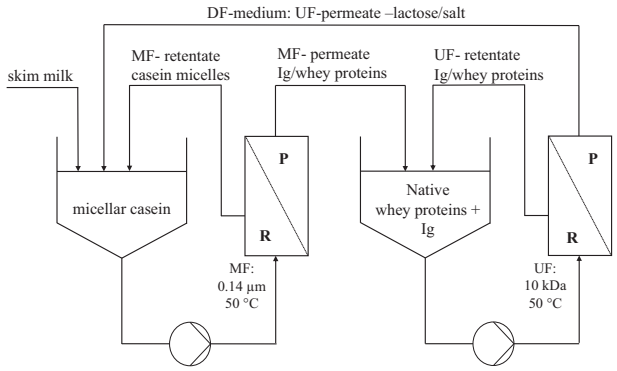

Fig. 1. Processing scheme for the fractionation of casein micelles and immunoglobulins operated in diafiltration mode using the protein free UF permeate as diafiltration liquid.

and composition of the native milk serum as well as pH nearly constant (6.5–6.55) and thus to avoid changes in the dynamic system of the casein micelles with the surrounding ionic strength or pH. Samples were collected during concentration and at each diafiltration step. After rinsing twice with 30 L of demineralised water, the alkaline and acidic cleaning of the MF system was carried out with 1% UF 466 and 0.5% nitric acid, respectively (Halag Chemie AG, Aadorf, Switzerland). The cleaning of the UF-system was done according to Piry et al. (2012).

2.2. Calculations of filtration performance

The reduction of the target substances in the MF retentate, which is described as the ratio of the residual content of the permeating components through the membrane at the end (C_{DF}) and the beginning ($C_{DF,0}$) of the DF process, is defined by equation (1). The exponential reduction depends on the transmission (p) and the number of diafiltration steps (DS), which is the ratio of the total volume of the diafiltration medium (V_{DF}) and the initial hold-up volume ($V_{DF,0}$). The transmission (p) is defined as concentration of a specific component in the permeate ($C_{i,Per}$) divided by the concentration of the same component in the retentate ($C_{i,Ret}$) at a given time (eq. (2)).

$$\frac{C_{DF,n}}{C_{DF,0}} = \exp(-p \cdot DS), \qquad DS = \frac{V_{DF}}{V_{DF,0}} \tag{1}$$

$$p = \frac{C_{i,Per}}{C_{i,Ret}} \tag{2}$$

When a component is not retained by either the membrane or in the deposited layer on its surface, p = 1. Values of p < 1 mean that partial retention has occurred either through the membrane or through a deposited layer of the retained casein micelles. Equation (3) defines the time for a diafiltration process as a function of the total amount of diafiltration medium (V_{DF}) and the permeate flow J.

$$t = \frac{V_{DF}}{J} \tag{3}$$

Combining equations (1) and (3) yields equation (4), which allows calculation of the required time (t) to remove a selected target component ($C_{t,DF}/C_{0,DF}$) from a volume $V_{0,DF}$ at the beginning of the DF process.

$$t = V_{0,DF} \times ln\left(\frac{C_{DF,n}}{C_{DF,0}}\right) * \frac{1}{p * J} \tag{4}$$

Prior to the operation in diafiltration mode, the skimmed milk ($V_{0,milk}$) was concentrated by a factor of two ($V_{DF,0}$), resulting in a reduction of the target components ($C_{0,DF}$) relative to the original amount of the respective component in the skimmed milk ($C_{0,milk}$) as defined by equation (5).

$$\frac{C_{0,DF}}{C_{0,\ milk}} = \frac{V_{0,}milk^{(1-p)}}{V_{DF,0}} \tag{5}$$

2.3. Isolation of Ig with size exclusion chromatography

IgG, IgA and IgM fractions were recovered by size exclusion chromatography using a Phenomenex Yarra 3 µm SEC4000 from the same colostral whey previously described by Heidebrecht et al. (2018a) to obtain pure fractions for the particle size measurement. The colostral whey was diluted by a factor of 50 with PBS buffer (Sigma–Aldrich, Irvine, United Kingdom) and 60 µL of whey were applied to the column. Isocratic elution was performed using 50 mM Na-phosphate buffer, pH 6.8, at a flow rate of 1 mL min^{-1}. Peaks were detected at 214 nm. To obtain a sufficiently high concentration of the Ig for the particle size measurement (IgG ≥ 0.96 mg mL^{-1}, IgA ≥ 0.36 mg mL^{-1}, IgM ≥ 0.16 mg mL^{-1}), 350 runs were pooled in PBS and concentrated to about 2 mL with Amicon® Ultra -10 kDa centrifugal filter devices according to the manufacturer's instructions. Peaks were identified by SDS-PAGE according to Holzmüller and Kulozik (2016).

2.4. Particle size analysis

The particle size distributions of the three Ig and the other whey proteins were determined to see if there is an overlap between the Ig fractions and the casein micelles. The particle size was measured by dynamic light scattering using the Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). To determine the particle size of the casein micelles skimmed milk was diluted 1:100 with lactose-free simulated milk ultrafiltrate according to Dumpler, Kieferle, Wohlschläger, and Kulozik (2017). Purified β-Lg (>99% in DM) and α-La (>92% in DM) powder were obtained as described elsewhere (Toro-Sierra, Tolkach, & Kulozik, 2013) and diluted with deionised water (Milli-Q Integral 3, Merck KGaA, Darmstadt, Germany) to a protein content of 1% and stirred for 12 h at 4 °C to ensure complete hydration. Pure lyophilised BSA and LPO were purchased from Sigma–Aldrich (St. Louis, MO, USA) and IgG, IgA and IgM were isolated as described above. The pH of all solutions was adjusted to 4.6 with HCl to precipitate non-native molecules. The sample was then centrifuged at 4000×*g* for 10 min. The supernatant was neutralised with NaOH to the natural milk pH of 6.8 and filtered using a syringe filter of 0.45 µm (Chromafil RC-45/25 Macherey–Nagel, Dueren, Germany). After 5 min equilibration phase, each sample was measured in duplicate at filtration temperature (50 °C). Each of the two runs consisted of 10 individual runs of 60 s.

2.5. Determination of IgG with reversed phase liquid chromatography

The quantitative determination of native or soluble IgG and α-La, β-Lg in one run was done by reversed phase-high performance liquid chromatography (RP-HPLC) using a PLRP-S 300 Å 8 µm column (Latek, Eppelheim, Germany) as described previously (Heidebrecht et al., 2018a). Prior to analysis, the pH of all samples was adjusted to 4.6 with 1 M HCl to precipitate casein micelles and

non-native whey proteins. In this context, the soluble whey protein concentration is considered equal to the native protein concentration (Marx & Kulozik, 2018). For the casein-rich MF-retentate samples the solution was centrifuged at 4000×*g*, 10 min, 4 °C to determine the concentration factor due to the precipitated caseins, which was depending on the casein concentration in the range of 1.08–1.2. There was no increase in concentration for the casein free MF-permeate and UF-retentate samples before and after centrifugation and thus this step was skipped. The supernatant of all samples was filtered through 0.45 μm syringe filters (Chromafil RC-45/25 Macherey–Nagel, Dueren, Germany).

2.6. Determination of denaturation degree with reversed phase liquid chromatography

To determine the degree of denaturation of α-La and β-Lg, the samples were additionally analysed by another RP-HPLC method described by Dumpler, Wohlschläger, and Kulozik (2017). The PLRP-S column (section 2.5) cannot be used, as caseins overlay the whey proteins in this method, which is why caseins are removed by pH 4.6 precipitation. However, at the same time denatured whey proteins are separated (Marx & Kulozik, 2018) and it is therefore not possible to determine the total protein whey protein concentrations. In the method according to Dumpler et al. (2017), however, the elution of caseins and whey proteins takes place one after the other. This makes it possible to determine the degree of denaturation (DD) by measuring the soluble protein concentration of the same sample in native format and after the adjustment to pH 4.6. The degree of denaturation (DD) is defined by equation (6), where C_A is sum of the respective soluble and insoluble whey protein concentration and C_N is only the soluble concentration

$$DD = \left(1 - \frac{C_N}{C_A}\right) \times 100\% \quad (6)$$

There was no difference between the native concentration of β-Lg as lead substance between the two methods (Fig. 2, Heidebrecht & Kulozik, 2019). Curves showing native α-La and β-lg are expressed as the average of the two independent methods. To determine the degree of denaturation raw skimmed milk (50, 55 °C) was pumped (1 bar TMP, 2 bar pressure drop, 6.2 m s^{-1}) through the MF membrane (see section 2.1) for 8 h and compared with the concentration of the respective proteins in the same milk, which was subjected to a tempered (50, 55 °C) water bath.

2.7. Determination of IgG, IgA and IgM with ELISA

Native IgG, IgA and IgM were determined using sandwich enzyme-linked immunosorbent assay (ELISA) test kits purchased from Bethyl Laboratories, USA (Cat. No. E10-118, E10-121 and E10-101). Samples were pre-diluted with a factor 1000–1500 for IgG and 200–300 for IgA and IgM with wash solution (E106) to stay in the calibration range of 500–7.8 ng mL^{-1} for IgG and 1000–15.625 ng mL^{-1} for IgA or IgM. The absorbance of duplicates at 450 nm was analysed using a Tecan Sunrise microtiter plate reader. The concordance correlation coefficient (Lin, 1989), which evaluates the equivalence of IgG values determined by ELISA and RP-HPLC, was 0.962 with a confidence interval of 95% from 0.946 to 0.974 (Fig. 2; Heidebrecht & Kulozik, 2019). A coefficient between 0.95 and 0.99 is considered as substantial agreement of a set of pairs of two measurements (McBride, 2005). Thus, the quantitative values of both methods are comparable. However, for consistency, the IgG data shown are only those measured by ELISA unless otherwise stated.

2.8. Data regression and statistical analysis

Linearised curves showing the decrease in MF concentration were fitted according to log linearised eq. (1). In addition, curves were fitted according to the subsequent equation, $C_{DF,n} = C_{DF,0} \times \exp(-p \times DS) + y_0$, which is equal to eq. (1) with the only difference being the y-offset $y_{,0}$. This causes a better correlation of the data at the beginning of the process, but limits the model to depletion levels of approximately 96–98%, depending on the offset. These data are depicted in Heidebrecht and Kulozik (2019). The process at 0.6 bar TMP was repeated three times with three separate batches of raw milk during several weeks. All other experiments were repeated at least twice. Data points represent the mean ± standard deviation. OriginPro 2017 software was used for all evaluations.

3. Results and discussion

3.1. Particle size of immunoglobulins

The goal of the MF fractionation process is to separate the casein micelles from the native whey proteins mainly due to the different particle size of these proteins. The particle size distributions of the whey proteins α-La, β-Lg, BSA, LPO, IgG, IgA, IgM and the casein micelles at filtration temperature of 50 °C are shown in Fig. 2 and the equivalent hydrodynamic diameters in Table 2. The mean hydrodynamic diameter of β-Lg was 4.19 nm and that of α-La was 2.29 nm. Similar mean diameters of 3.7 nm (Dombrowski, Dechau, & Kulozik, 2016) and 4.5 nm (Gebhardt, Toro-Sierra, & Kulozik, 2012) for native β-Lg at natural pH and α-La 1.91 nm (Gast, Zirwer, Müller-Frohne, & Damaschun, 1998) were reported previously at 20 °C. For IgG the maximum of the particle size distribution was 10.67 nm, consistent with results from Le Berre and Daufin (1998). For dimeric secretory IgA, the D_{50} was 18.09 nm and the pentamer IgM had a value of 23.78 nm, with 90% of the particles being less than 24.79 nm and 29.78 nm, respectively. Fig. 2 also shows that there is no overlap between the particle size distributions of the immunoglobulins and the casein micelles. Theoretically, it should therefore be possible to separate the proteins from each other by means of a filter membrane.

3.2. Fractionation of casein micelles and immunoglobulins with MF in diafiltration mode

The aim was to assess the filtration behaviour of the Ig fractions with a typical MF-membrane (nominal pore size 0.14 μm) for milk protein fractionation and to separately measure the transmission and yield factors for all individual Ig fractions. Fig. 3 shows the

Table 2
Particle diameter (in nm) of different whey proteins.[a]

Parameter	α-La	β-Lg	BSA	LPO	IgG	IgA	IgM	Casein
D 10	1.75 ± 0.18	3.14 ± 0.23	6.43 ± 0.59	6.44 ± 0.52	7.86 ± 0.48	13.86 ± 0.68	19.29 ± 1.99	112 ± 15
D 50	2.29 ± 0.12	4.19 ± 0.2	7.82 ± 0.68	8.16 ± 0.5	10.67 ± 0.4	18.09 ± 0.78	23.78 ± 2.7	182 ± 27
D 90	3.06 ± 0.16	5.85 ± 0.18	9.58 ± 0.80	11.78 ± 1.37	15.33 ± 0.92	24.79 ± 1.00	29.78 ± 3.6	300 ± 42

[a] D10, D50, D90 = 10%, 50%, 90%, respectively, of the particles are smaller than the given value.

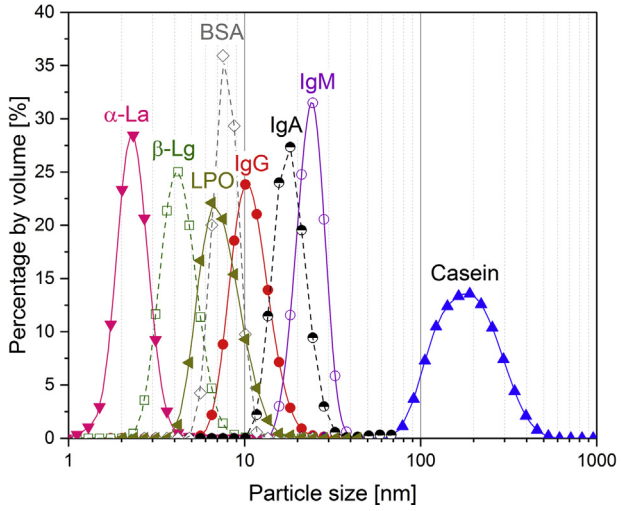

Fig. 2. Particle size distribution of native whey proteins and casein micelles. Abbreviations are: α-La, α-lactalbumin; β-Lg, β-lactoglobulin; LPO, lactoperoxidase; BSA, bovine serum albumin; IgG, immunoglobulin G; IgA, immunoglobulin A; IgM, immunoglobulin M (n = 2; of 10 measurements each).

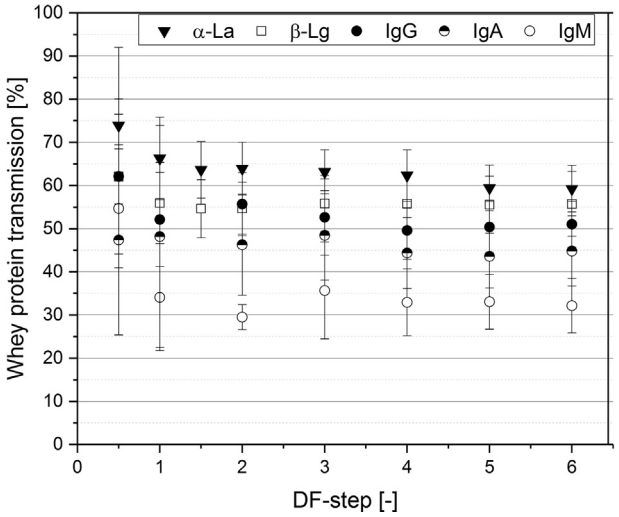

Fig. 4. Transmission of immunoglobulins and major whey proteins as function of the number of DF-steps at TMP of 0.6 bar, n = 3: ▼, α-lactalbumin; □, β-lactoglobulin; ●, immunoglobulin G; ◓, immunoglobulin A; ○, immunoglobulin M.

decrease of α-La, β-Lg, IgG, IgA and IgM in the MF-receiving tank compared with the ideal decrease at 100% transmission and the decrease of the casein micelles as a function of the DF steps. The curves were log-linearised and fitted according to eq. (1) (Table 1, Heidebrecht & Kulozik, 2019). In addition, curves were fitted according to eq. (1) with the only difference of containing and a y-offset y_0 (Table 2, Heidebrecht & Kulozik, 2019). This causes a better correlation of the data at the beginning of the process, but limits the model to depletion degrees of approximately 96–98%, depending of the offset; the second dataset is given in brackets (for details see Heidebrecht & Kulozik, 2019).

As can be seen, the casein concentration remains constant, while the concentration of all whey proteins decreases due to the washout effect of the MF. Compared with the ideal decrease at p = 1, the slope of all curves is less steep, i.e., p < 1. Although the mean pore diameter of the membrane is 0.14 μm and thus far greater than all whey proteins, the transmission of the native proteins is not 100% but in the range of p = 0.3–0.8 or 30–80% (Fig. 4). This is mainly due to the

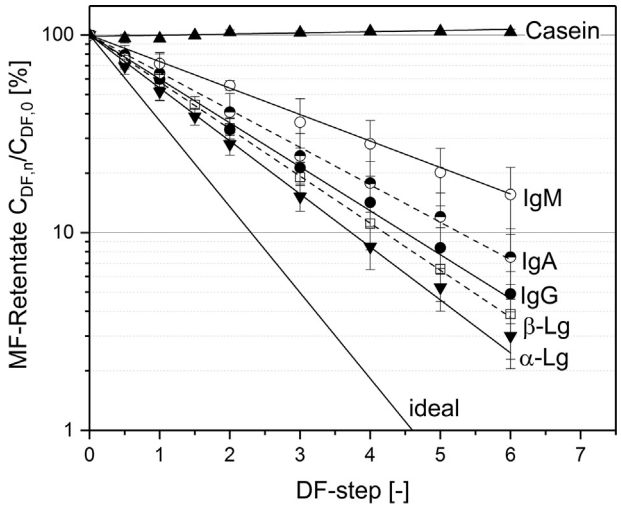

Fig. 3. Decrease in the MF-receiving tank of α-lactalbumin (α-La), β-lactoglobulin (β-Lg), immunoglobulin G (IgG), immunoglobulin A (IgA) and immunoglobulin M (IgM) in comparison with the ideal decrease at 100% transmission and the decrease of the casein micelles as a function of the number of DF-steps at TMP of 0.6 bar, n = 3.

retention of casein micelles, which form a deposit on the membrane surfaces and thus act as a secondary separation layer with its own retention capacity (Le Berre & Daufin, 1996; Piry et al., 2008). For α-La, 3.8 (3.7) DF-steps are required, and for β-Lg 4.2 (4.2) DF-steps, to reduce the concentration by 90%. In other words, this means 10% of the initial concentration, relative to the start of the DF, remains in the MF feed tank. These results are in the expected range based on previous data during batch experiments (Piry et al., 2008). For a 90% removal of the immunoglobulins from the MF-retentate, there are 4.6 (4.6) DF-steps necessary for IgG, 5.3 (5.4) DF-steps for IgA, and 7.3 (7.5) DF-steps for IgM, which implies that the porosity of the casein deposit layer is open enough to allow the transmission of the immunoglobulins. As previously reported, there is almost no difference in the transmission of IgG and β-Lg (Heidebrecht et al., 2018b), although IgG is about 2.5-fold greater based on the mean hydrodynamic diameter. A possible explanation for the good transmission of IgG could be the lack of or little electrostatic interactions between the proteins and the deposited casein micelles. At a filtration pH of 6.5–6.55 at 50 °C casein micelles are negatively charged while IgG nearly neutral or slightly negative. IgG occurs in milk in the two subclasses IgG_1 (IEP 5.5–6.8) and IgG_2 (IEP 7.5–8.3), of which IgG_1 accounts for about 90% of the total IgG fraction (Marnila & Korhonen, 2011). The IEP of total IgG (IgG_1 and IgG_2, as determined in this study) was measured to be 5.8–6 (Fig. 3, Heidebrecht & Kulozik, 2019). Therefore, at a filtration pH of 6.5, IgG nearly neutral or weakly negative, resulting in little electrostatic repulsion between IgG and the deposited casein micelles, which in turn could positively affect transmission. This explanation is in agreement with (Le Berre & Daufin, 1998), who showed that IgG transmission is independent of the ionic strength, unlike the other whey proteins, where transmission increased with increasing ionic strength. The given rational was the reduction of electrostatic interactions. Beneficial transmission at a filtration pH close to the IEP has also been reported for other whey proteins such as BSA (Persson, Jönsson, & Zacchi, 2003).

For bovine IgM, the IEP was 4.7 (Fig. 3, Heidebrecht & Kulozik, 2019), which is in the range of data reported for human IgM (4.5–6.5) (Luo, Zhang, Yao, & Lin, 2018). For IgA, we could not measure the IEP because there was not enough material for the measurement. However, its IEP is expected to be similar or slightly higher than IgM based on data for human IgA (4.5–6.8) (Luo et al., 2018). Thus, IgM and IgA should be negatively charged similar to the major whey proteins α-La (IEP 4.2–4.5) and β-Lg (IEP 5.13), and their

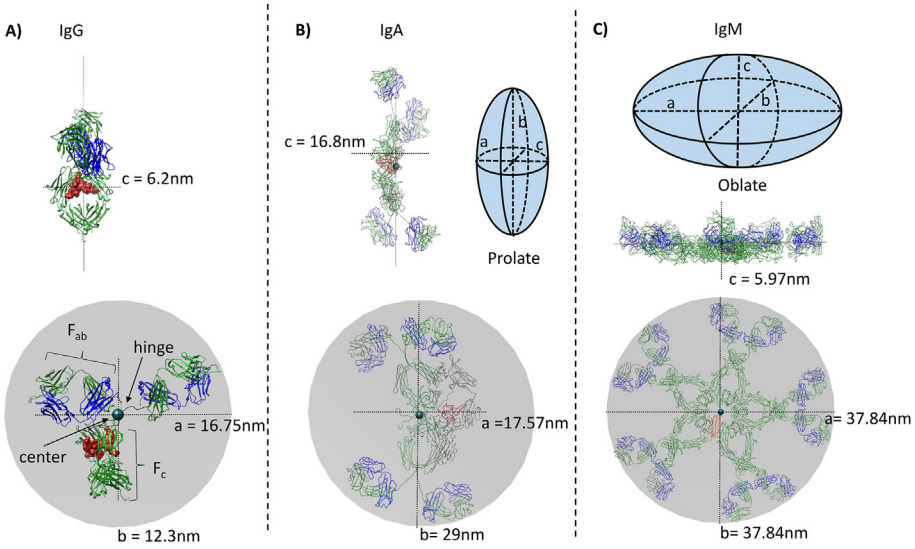

Fig. 5. Shape and size of different immunoglobulins. Three dimensional structure generated with UCSF Chimera (Pettersen et al., 2004), using pdb code 1HZH (IgG) (Saphire et al., 2001), 3CHN (IgA) (Bonner, Almogren, Furtado, Kerr, & Perkins, 2009), 2RCJ (IgM) (Perkins, Nealis, Sutton, & Feinstein, 1991) from RCSB Protein Data Bank. Prolate (two short sides, one long side), oblate: (two long sides, one short side).

transmission is expected to be more dominated by steric exclusion. Nevertheless, the flexibility and shape of the proteins could also contribute to their still relatively good transmission. A single Ig monomer consist of two identical antigen-binding (Fab) regions and a constant (Fc) region linked by a hinge region with no secondary structure, which gives the molecule a high degree of flexibility (Fig. 5A). It has been reported that prolate-like particles contribute less to the specific fouling resistance compared with spherical particles, whereas oblate particles have a greater impact (Endo, Chen, & Pui, 2002; Endo, Ngan, Nandiyanto, Iskandar, & Okuyama, 2009). The shape of the three immunoglobulins is schematically depicted in Fig. 5. Dimeric secretory IgA has a prolate-like shape and IgM an oblate-like shape which, in addition to the size difference, might contribute to the comparatively lower performance of IgM to IgA. In summary, however, it was possible to separate casein micelles and all immunoglobulins in bovine milk by MF.

3.3. Required time to achieve a certain whey protein decrease in the MF retentate

Eq. (4) shows that the required time for a filtration process operated in DF mode decreases when either the initial volume $V_{0,DF}$ or the desired degree of purity decreases, or, on the other hand, as the transmission and/or flux increase. The conclusion would theoretically be to concentrate the skimmed milk before the DF process as much as possible to reduce $V_{0,DF}$. The well-known downside is that the flux decreases with increasing concentration factor (Kulozik & Kersten, 2002), which is why the skimmed milk was concentrated only by a factor of two (Reitmaier, Heidebrecht, & Kulozik) before the start of the DF process. However, during the concentration phase, the whey proteins already pass through the membrane (eq. (5). Thus, the absolute amount decreases before the process is run in DF mode. Fig. 6 (Tables 3 and 4, Heidebrecht & Kulozik, 2019) shows the absolute decrease of α-La, β-Ig, IgG, IgA and IgM for the concentration plus diafiltration phase, based on the absolute amount in the 50 L of the skimmed milk used as a function of the time. The time was normalised to 1 m^2 of membrane area by multiplying the actual time by the actual membrane area (0.35 m^2). For IgG it takes 84.6 min, for IgA 119.2 min and for IgM 160.4 min to reduce the initial concentration by 90%. The time increases greatly by 25–60% when the desired degree of removal is increased to 95%. These data are valuable for the layout and the scale-up of filtration plants. For example, if the aim is to extract 90% of IgG from 1000 L of skim milk instead of 50 L as in this study, the time would increase by a factor of 20 for 1 m^2 of membrane area. Therefore, it would make sense to increase the filtration area to 20 m^2 to achieve the same effect in the same time.

3.4. Impact of transmembrane pressure on the fractionation performance

Gradient membranes are engineered to balance the inhomogeneous pressure conditions along a membrane and thus improve the overall filtration performance of a membrane (Skrzypek & Burger, 2010). The gradient membranes used are designed with a

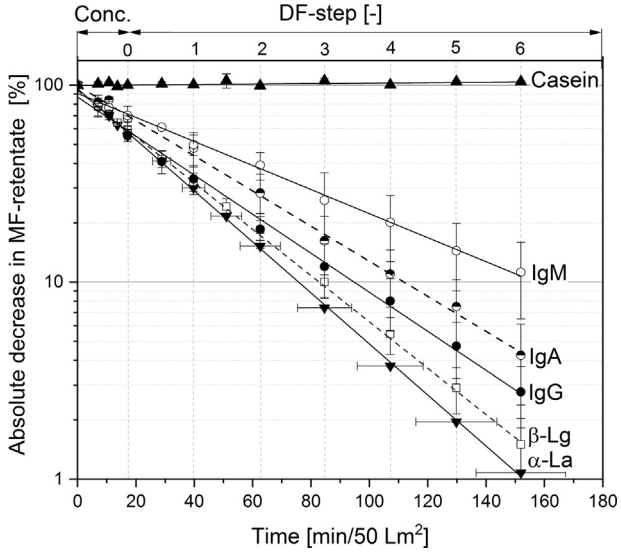

Fig. 6. Decrease in the MF-receiving tank of α-lactalbumin (α-La), β-lactoglobulin (β-Lg), immunoglobulin G (IgG), immunoglobulin A (IgA) and immunoglobulin M (IgM) as function of time at TMP of 0.6 bar. The time normalised to 1 m^2 of membrane area and 50 L of skim milk, n = 3.

fixed, longitudinally decreasing membrane resistance, which is realised by reducing the thickness of the selective layer along the membrane. This decreasing membrane resistance is proportional to the unavoidable loss of retentate pressure with the purpose of creating a constant flux along the membrane. However, to achieve this isoflux and better overall performance compared with conventional membranes, the gradient membrane with the fixed membrane resistance requires a defined retentate pressure drop or crossflow rate, respectively. For a given membrane and temperature, the TMP remains the key variable to optimise the process. Fig. 7 shows the transmission as function of the DF steps at different TMP for α-La, β-Lg, BSA, IgG, IgA and IgM. In general, transmission was constant throughout the DF process, independent of the applied TMP and protein considered. Only for a TMP of 0.6 bar there was a slight decrease from DF 0.5 to DF 1. The reason is likely to be that the time to reach a near steady state between deposit layer formation and removal is longer at a lower TMP.

Contrary to the expectation and to previous reports (Piry et al., 2012), there were only minor differences in transmission among the various TMPs. Only at a TMP of 0.6 bar, the transmission was slightly higher and at a TMP of 3 bar, the transmission was slightly lower than the other values. Transmission was determined both by solving the equation (1) after transmission (for MF decrease data see Fig. 4, Heidebrecht & Kulozik, 2019) and by equation (2) as the concentration ratio of a specific component in the permeate and the concentration of the same component in the retentate at a given DF step. The results determined by eq. (2) were somewhat higher, but in both methods there was no dependence on the TMP (Fig. 5, Heidebrecht & Kulozik, 2019). One possible explanation is that all TMPs have reached a limiting range in terms of deposit layer formation and deposit layer removal with respect to transmission. In this case, the higher compaction of the deposit layer on the membrane surface layer, plays a minor role.

The order of transmission of the individual whey proteins was α-La > β-Lg > IgG > LPO > IgA > IgM > LF > BSA, regardless of the TMP used (Fig. 8). The mean transmission across all TMP and data points was 55% > 50%> 47% > 41%> 39% > 32%> 22% > 19% of the respective proteins. This order shows that the transmission does not depend exclusively on steric exclusion (compare Fig. 2), but other factors must also contribute to the transmission of the individual proteins. Such mechanisms might be the architecture of the proteins and electrostatic interaction between the individual proteins and the deposited casein micelles, as discussed in section 3.2. A possible explanation for the comparatively poorer performance of the positively charged proteins LF with an IEP of 8.8 (Farrell et al., 2004) and LPO with an IEP of 9.6 (Farrell et al., 2004) is that the

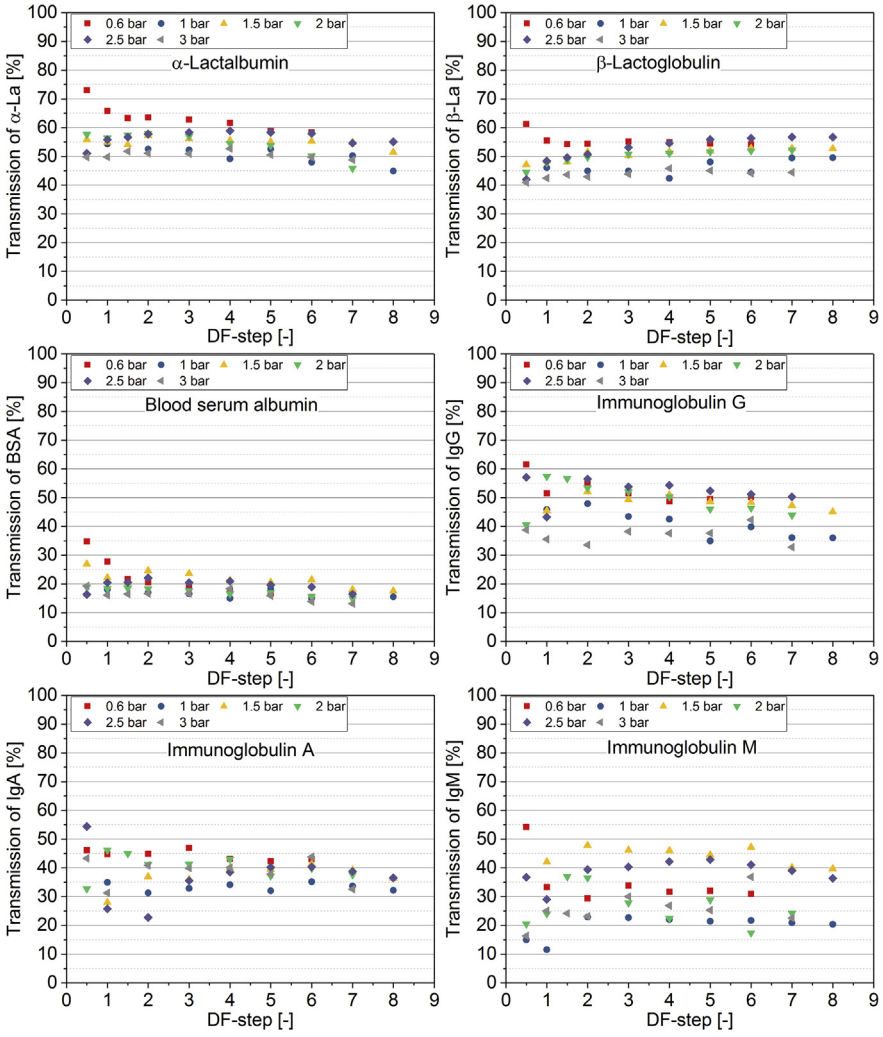

Fig. 7. Transmission of immunoglobulins, BSA, and major whey proteins as function of the number of DF-steps at indicated TMP: ■, 0.6 bar; ●, 1 bar; ▲, 1.5 bar; ▼, 2 bar; ◆, 2.5 bar; ◀, 3 bar. n = 2, except 0.6 bar n = 3.

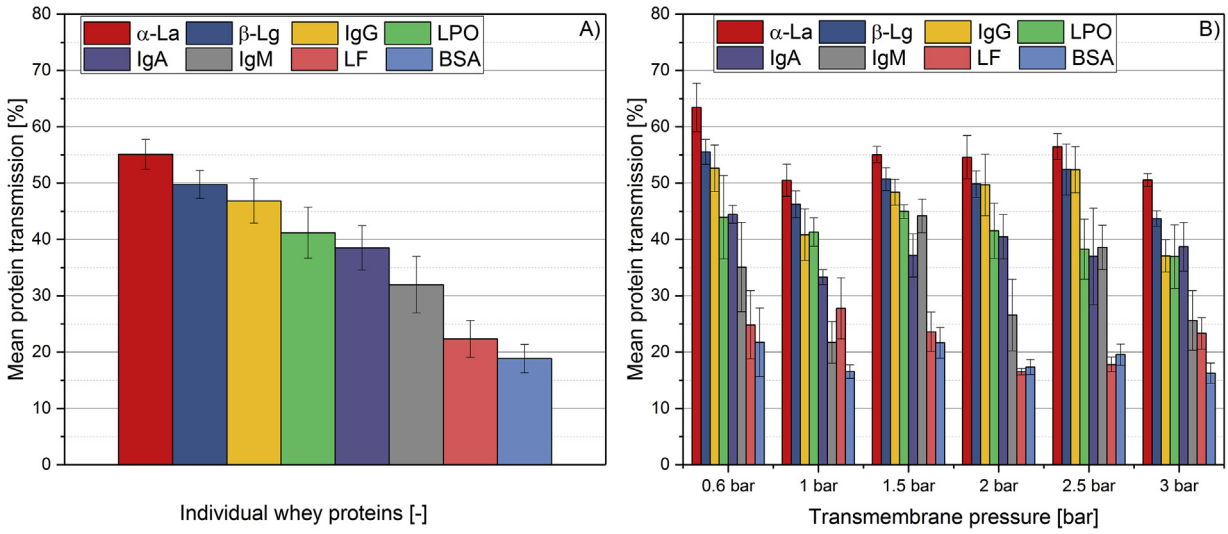

Fig. 8. Mean individual protein transmission of all data points (A) as function of time and TMP and (B) (as function of time) at different TMP. Mean for LF and LPO was only from DF 0.5–2. Abbreviations are: α-La, α-lactalbumin (■); β-Lg, β-lactoglobulin (■); IgG, immunoglobulin G (■); LPO, lactoperoxidase (■); IgA, immunoglobulin A (■); IgM, immunoglobulin M (■); LF, lactoferrin (■); BSA, bovine serum albumin (■).

proteins are entrapped in the casein micelle deposit, but this is not a valid explanation for the inferior performance of BSA with an IEP between 4.7 and 4.9 (Farrell et al., 2004). Studies on BSA during cross-flow filtration showed that the shear flow leads to protein aggregates that subsequently deposit on the membrane surface (Kim, Chen, & Fane, 1993). Moreover, thiol-interchange could be responsible for the poorer transmission due to the one free sulfhydryl group of BSA (Kelly & Zydney, 1994).

Regarding protein transmission, there was a tendency that at 0.6 bar the transmission for the individual proteins was higher. However, the time to achieve a 90% removal relative to the initial concentration in the skimmed milk was shorter at a working TMP of 1 and 1.5 bar compared with 0.6 bar (Fig. 6, Heidebrecht & Kulozik, 2019). The reason for this was the higher flux, which was about 100 L h^{-1} m^{-2} at a TMP of 1 bar, 80 L h^{-1} m^{-2} at 1.5 bar and only 65 L h^{-1} m^{-2} at 0.6 bar (Fig. 7, Heidebrecht & Kulozik, 2019). Pertaining to the operating pressure, the shortest process time for a 90% removal for α-La, β-Lg and IgA was at 1 bar and for IgG and IgM at 1.5 bar.

In summary, contrary to the hypothesis that a higher TMP pushes the flexible molecules through the membrane and deposit layer, there was little dependence on the transmission. Thus, the shortest process time to achieve complete separation for the individual proteins was primarily controlled by the TMP leading to the highest flux.

3.5. Required time to achieve a certain yield in the native whey

The presented data so far showed a reduction of the antibodies from skimmed milk in the MF-retentate. However, it seems also or even more relevant to assess the increase of the proteins in the UF-retentate (Fig. 9; for TMP-dependent data see Fig. 8, Heidebrecht & Kulozik, 2019). Therefore, in addition to the decrease in MF, the increase in concentration in the UF feed tank was measured. The measurement serves as an independent control to confirm that the minor fractions are transferred in the whey in native format and, for example, are not precipitated or entrapped in the deposit. The reason why only MF data were presented before section 3.5 is that the determination of the absolute decrease in the MF-feed tank is more accurate than the determination of the absolute increase in whey. The data shown are equivalent to a mass balance, which was additionally normalised to a defined filter area and initial volume of milk. The absolute decrease (Fig. 6) or increase (Fig. 9) of the concentration ($C_{t,abs.}$) in the MF- or UF-feed tank, is calculated by multiplying the measured concentration (C_t) by the corresponding volume (V_t). This value is and related to the absolute amount in the initial quantity ($C_0 \times V_0$) (eq. (7)).

$$C_{t,\,abs.} = \frac{C_t \cdot V_t}{C_0 \cdot V_0} \tag{7}$$

The initial amount in the skimmed milk and, accordingly, the reference value is the same for both graphs. In contrast, $C_{t,MF}$

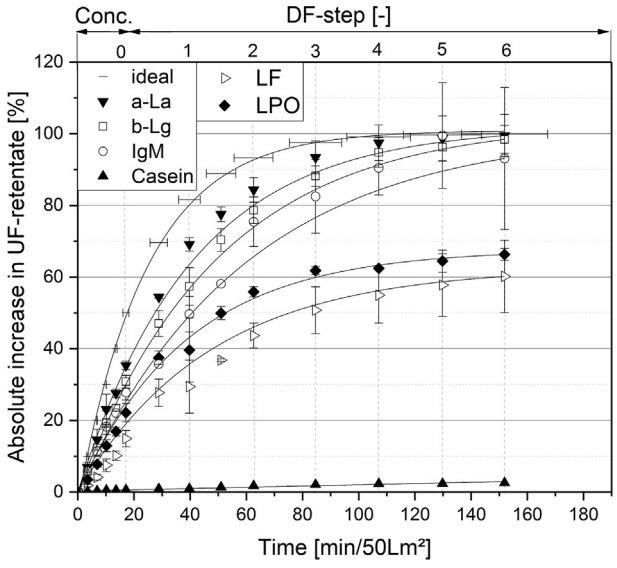

Fig. 9. Measured concentration increase in the UF-receiving tank of α-lactalbumin (▼), β-lactoglobulin (□), immunoglobulin M (○), lactoperoxidase (◆) and lactoferrin (▷) in comparison with the ideal decrease (–) at 100% transmission as a function of time based on 1 m^2 membrane area and 50 L skim milk (▲, casein). The time standard deviation was drawn in the ideal curve instead of at each measured data point to maintain clarity of the diagram, n = 3.

decreases and $C_{t,UF}$ increases during the filtration process operated in DF-mode. Even though the transmission is constant throughout the entire filtration process in DF-mode (Fig. 4), the total concentration increase (C_t) is marginal after about 3–4 DF steps in the UF feed tank. For example, in DF 5, the β-Lg concentration in the MF-retentate is about 0.2 mg mL^{-1} and in the UF-retentate 8 mg mL^{-1}. In DF 6, the concentration in the MF-retentate is about 0.1 mg $mL^{-1}$and in the UF-retentate 8.1 mg mL^{-1}. For the concentration in the UF-feed tank, this means that minor analytical errors (<3%) dominate the slight increase in concentration, as indicated by the high standard deviation in Fig. 9, and it is therefore difficult to derive models. In this example, this would mean that the measured value would be 8.1 ± 0.24 mg mL^{-1}. The analytical error is therefore higher than the actual increase. The analytical error is increased by required analytical dilution and multiplication with high volumes of whey. In contrast, the measured concentration in the MF-retentate in the given example would be 0.1 ± 0.003 mg mL^{-1}. Thus, the analytical error plays a minor role.

In conclusion, even if the interest is in the increasing protein concentration in the whey, it is more precise to calculate the increase in the UF feed tank using the measured values in the MF retentate and subtract the values from the initial total concentration than to measure the progress of increase in concentration in the UF feed tank. Thus, the increase of the individual proteins in the whey (UF-retentate) is reciprocal to the decrease of the corresponding proteins in the MF retentate and can be calculated with the same parameters from Tables 1–4 in Heidebrecht and Kulozik (2019), changing only the negative sign to a positive sign. However, the opposite is true for the detection of casein-induced impurities. Although no casein loss was found in the MF retentate (Fig. 3) and casein transmission was unquantifiable, there was a slight but measurable (1–2%) cumulative increase in the whey due to the transmission of casein monomers. This is in the range of previously reported data (Adams & Barbano, 2013). In addition, it was also possible to measure the increase in LF and LPO in the UF that were below the analytical detection limit from DF 2 in the MF-retentate, and to confirm their low transmission shown in section 3.4.

3.6. Impact of process stress on the nativity of the whey proteins

In addition to a high yield, it is essential to maintain the nativity to avoid denaturation of the obtained proteins over longer filtration times. The filtration time for milk protein fractionation at 50 °C is limited to about 8 h due to the growth of thermophilic microorganisms. The question was whether the long-term process stress at 50 °C or 55 °C as possible alternative filtration temperature and the high flow velocities (6 m s^{-1}) affects the nativity of the proteins and, if so, whether the MF filter membrane retains the insoluble proteins. To answer the first question, raw skimmed milk was pumped (2 bar pressure drop, 6 m s^{-1}) over the MF membrane for 8 h while permeate and retentate were directed into the MF feed tank, creating a closed loop. The potential effect of temperature and shear was differentiated by additionally exposing the same milk to a water bath and therefore only to the impact of the respective temperature. However, neither only 50 °C (Fig. 10) or 55 °C (Fig. 9, Heidebrecht & Kulozik, 2019) nor the particular temperature and shear stress during batch filtration resulted in a reduction of soluble IgG and α-La. However, there was slight impact on β-Lg (3–4% denaturation, 50 °C), which increased to approximately 15% at 55 °C (Fig. 9, Heidebrecht & Kulozik, 2019). In summary, microfiltration at 50 °C or 55 °C had no effect on the nativity of IgG but did to a certain extent on β-Lg. The second question was therefore, whether the insoluble β-Lg proteins are retained by the membrane during diafiltration. These data are shown in the aligned Data in Brief publication (Heidebrecht & Kulozik, 2019).

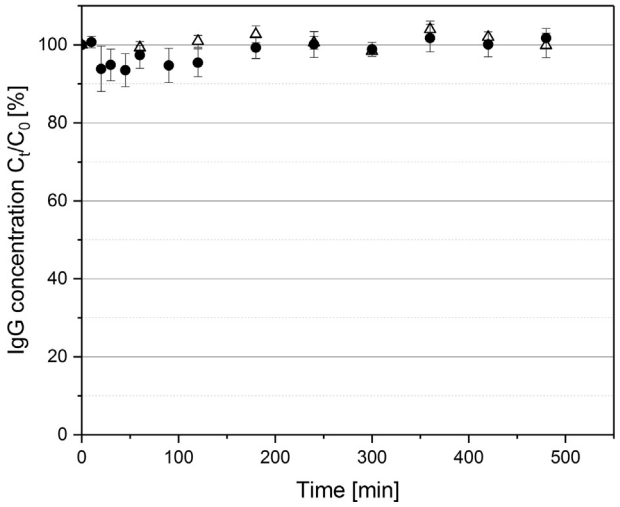

Fig. 10. IgG concentration (RP-HPLC) in skim milk during batch filtration (●) compared with IgG concentration in a water bath (△) at 50 °C for 8 h, n = 3.

In summary, long-term process stress leads to insignificant losses of IgG and α-La, but results in a minor denaturation of β-Lg. However, these non-native proteins are selectively retained by the MF-membrane, so that the resulting whey contains only native proteins. Another conclusion is that it is also possible to produce high quality native whey from gently heat-treated milk, since the aggregated proteins cause by heat pretreatment would be retained. Even though denaturation was marginal, it points out that there is a trade-off between high yield and minimal degree of denaturation, which should be carefully considered for a given aim. A practical way to minimise this trade-off is to adjust the membrane area for a given volume to a maximum filtration time and a minimum yield target.

4. Conclusion

The mean hydrodynamic diameter was measured for the three immunoglobulins IgG, IgA and IgM in milk at 10.7 nm, 18.1 nm and 23.8 nm Therefore, although they are 2.5–10.5 times larger than the major whey proteins α-La (2.3 nm) and β-Lg (4.2 nm), it was possible to fractionate casein micelles and immunoglobulins by MF operated in DF mode and to obtain the Ig fractions together with the other smaller whey proteins in the MF filtrate of a 0.14 μm membrane. The order of transmission of all individual whey proteins was α-La> β-Lg > IgG > LPO (8.2 nm)> IgA > IgM > LF > BSA (7.8 nm), independent of the applied TMP, indicating that the transmission does not depend only on the size of the whey proteins. Due to the insignificant TMP dependence on the transmission, the shortest process time to achieve complete separation of the individual proteins was primarily controlled by the TMP (1–1.5 bar) leading to the highest flux. The average transmission of immunoglobulins varied between 32 and 49%. After preconcentration by factor of two, with these levels of transmission, 90% of the initial IgG, IgA and IgM could be recovered within 84, 119, and 160 min, based on 1 m^2 of membrane area and 50 L of skimmed milk. In conclusion, the use of MF is applicable not only for the fractionation of casein micelles and the major whey proteins. but also for the fractionation of caseins and immunoglobulins, which was previously unknown for IgA and IgM. The data can be used to design filtration systems for a given amount of feed in terms of required membrane area, thereby enabling large-scale production of immunoglobulin rich native whey. In addition, it has been shown that long-term process stress has no effect on IgG

and α-La and only marginal impact on the denaturation of β-Lg. However, the small portion of denatured β-Lg is selectively retained by the MF-membrane and its deposit layer so that the whey obtained contains only native proteins. In summary, it is possible to use long-term MF (8 h) in DF mode for enriching the individual Ig from the two perspectives of yield as well as maintenance of nativity.

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