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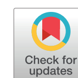
Isolation of biofunctional bovine immunoglobulin G from milk- and colostral whey with mixed-mode chromatography at lab and pilot scale



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ABSTRACT

The aim of the present work was to develop a new scalable and cost-efficient process to isolate bovine immunoglobulin G from colostral whey with high purity and minimal loss of activity. The mixed mode material Mercapto-Ethyl-Pyridine-Hypercel^{TM} was identified appropriate for direct capture of immunoglobulin G. The binding mechanism is primarily based on hydrophobic interactions at physiological conditions. As compared to immunoglobulin G, all other low molecular whey proteins such as α-Lactalbumin or β-Lactoglobulin, except lactoperoxidase, are more hydrophilic and were therefore found in the flow-through fraction. In order to remove lactoperoxidase as an impurity the column was combined in series with a second mixed mode material (Capto^{TM}- with N-benzoyl-homocysteine as ligand) using the same binding conditions. At pH 7.5 the carboxyl group of this ligand is negatively charged and can hence bind the positively charged lactoperoxidase, whose isoelectric point is at pH 9.6. After sample application, the columns were eluted separately. By combining the two columns it was possible to obtain immunoglobulin G with a purity of >96.1% and yield of 65–80%. The process development was carried out using 1 mL columns and upscaling was performed in three steps up to a column volume of 8800 mL for the Hypercel^{TM} column and 3000 mL for the Capto^{TM}- column. At this scale it is possible to obtain 130–150 g pure immunoglobulin G from 3 L colostrum within five hours, including the regeneration of both columns. Additionally, the impact of freeze-drying on the isolated immunoglobulin G was studied. The nativity of the freeze dried immunoglobulin was above 95%, which was proven by reversed phase liquid chromatography and validated by differential scanning calorimetry. The activity of immunoglobulin G was preserved over the isolation process and during drying as measured by enzyme-linked immunosorbent assay. In conclusion, by applying the proposed isolation process, it becomes feasible to obtain pure, active and stable imunnunoglobulin G at large scale.

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1. Introduction

Colostrum is the first milk produced by the cow after parturition. Since the maternal immunoglobulin cannot passage the cow's placenta the new born calf does not have an own effective humoral immune system directly after birth. Hence, it has to receive its immunity through the mother's milk. Therefore the total immunoglobulin concentration with 20–200 mg mL^{-1} is naturally very high in colostrum and renders it a perfect starting material for the isolation of the major immunoglobulin class G (IgG) (15–180 mg mL^{-1}) [1]. Its natural function is to opsonize and agglutinate pathogens and initiate the classical complement pathway as well as neutralize soluble virulence factors, which makes it an interesting material for various food supplements and pharma applications [2].

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Colostrum naturally contains antibodies directed against various pathogens. It shows a significant antimicrobial activity against *Staphylococcus aureus* [3], which is one of the major pathogens causing cow's mastitis but is also involved in human skin diseases such as atopic dermatitis and impetigo contagiosa [4]. Current treatment consists of topically applied antibiotics with the risk of antibiotic resistant bacterial strains and destruction of the healthy skin microflora [5].

Based on the anti-*Staphylococcus* activity of colostrum an alternative therapeutic concept is conceivable. Isolated IgG may be used to specifically target pathogenic bacteria in bacterial skin infections without affecting the healthy microflora and causing the development of therapy resistance. The specific titer of immunoglobulins in colostrum against a certain pathogen such as *Staphylococcus aureus* can be increased by immunizing the cow as described for other pathogens such as enterotoxigenic *Escherichia coli* [6,7], *Clostridium difficile* [8,9], *Shigella flexneri* [10] or rotavirus [11]. Prerequisite of a development of IgG isolated from colostrum as drug substance for therapeutic use, is the standardized production of pure and active IgG at an industrial scale.

One of the most frequently used methods for the isolation of bovine IgG is affinity chromatography using different types of chromatography matrices and ligands such as metal chelate interaction chromatography with copper ions [12–14] or zinc ions [15], immunoaffinity chromatography with IgY antibodies from egg yolk as ligand [16,17], protein G [18,19] or thiophilic chromatography [20,21]. Due to the high molecular weight of IgG of 150–161 kDa compared to the other whey proteins α-lactalbumin (α-La, 14 kDa), β-lactoglobulin (β-Lg, 18 kDa), blood serum albumin (BSA, 66 kDa), lactoferrin (LF, 76 kDa), lactoperoxidase (LPO, 79 kDa) size exclusion is another applied method [22,23]. However, even though these techniques lead to a high purity in general, they are difficult and costly to scale up. Due to the wide isoelectric point range of IgG from 5.5 to 8.3 ion-exchange chromatography leads to a low purity when used as a single column [24,25] or to a low yield when used in series [26].

A rather new mixed-mode matrix first reported by Burton and Harding [27] is the hydrophobic charge-induction chromatography (HCIC) where the binding takes place under physiological conditions which makes this method suitable for bioactive and sensitive proteins [28]. A key feature of HCIC is that adsorption and desorption of the proteins to the column rely on different mechanisms. At neutral pH the binding takes place by hydrophobic interactions, whereas at low pH the elution mechanism is based on electrostatic repulsion. The salt independency of this technique offers advantages for large-scale applications so that ideally the feed can be loaded onto the column without the addition of salts or change of the pH [29].

HCIC using 4-mercapto-ethyl-pyridine (MEP) as ligand has been reported as an effective method for the separation of antibodies from a variety of feedstocks such as cell culture supernatant and crude ascites [30–34]. This ligand was reported as useful by Wu et al. [35] to isolate IgG from colostrum, but with a limited purity due to a significant content of β-lg and without showing the scalability of the process and monitoring the activity of IgG.

In summary, obtaining high amounts of pure and active IgG isolated from colostrum by means of scalable processes remains a challenge. Hence, the aim of this work was to develop a new scalable and cost-efficient process to isolate IgG from colostrum with high purity and recovery and minimal loss of activity. The hypothesis was that the purity of the finally obtained IgG could be increased to more than 95% and that the scale-up could be enabled by a factor higher than 1000. The approach applied in this study was to combine two scalable mixed mode materials. The binding conditions were adjusted to each other to allow serial connection while

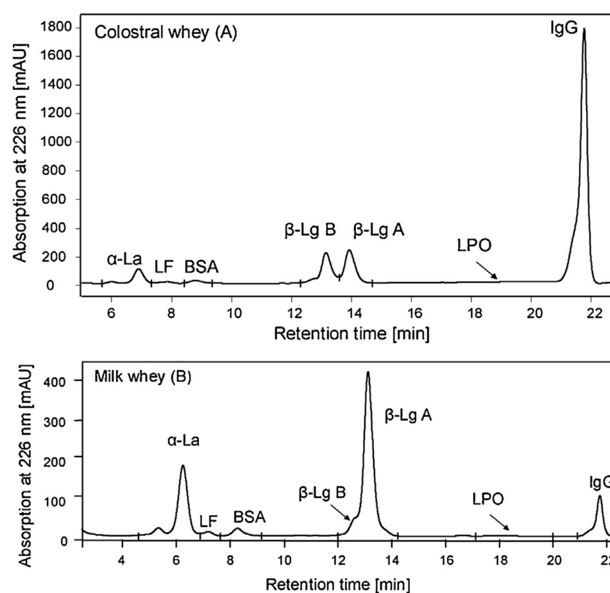

Fig. 1. Chromatograms of the obtained native whey from colostrum (A) and milk (B). α-La: α-lactalbumin, LF: lactoferrin, BSA: blood serum albumin, β-Lg: β-lactoglobulin (genetic variants A and B), LPO: lactoperoxidase, IgG: immunoglobulin G.

applying comparatively gentle binding and elution conditions to preserve the nativity and activity of the IgG.

2. Experimental

2.1. Whey preparation from colostrum and milk

Even though the main objective was to finally isolate IgG from colostral whey, at first we used whey obtained from mature milk as model liquid for the process development. In colostral whey, IgG represents 60–80% of the total whey protein fraction, in milk whey only 5–15%. Thus, it is easier to detect impurities due to the different ratio of IgG to other whey proteins in isolates derived from milk (see Fig. 1).

To avoid thermally induced changes of the immunoglobulins frozen raw colostrum (first and second milking) was purchased from the colostrum processor Colostrum Biotech (Königsbrunn, Germany). Raw milk was collected directly from a local farm and frozen at −18 °C in food grade plastic containers to be able to store the milk for an extended period of time. To thaw the milk or colostrum, the boxes were put in a cold water bath at 4 °C for 12–14 h whereat the product temperature never exceeded 4 °C. The milk was separated with a pilot cream separator type MM 1254 D (GEA Westfalia, Oelde, Germany) at 8000 g and 50 °C to achieve a fat content below 0.1% in the skim milk and colostrum. The casein micelles were fractionated from the whey proteins by microfiltration as described by Piot et al. [36]. The microfiltration unit was coupled with an ultrafiltration (cut off 10 kDa) unit and operated in diafiltration mode using the same equipment as shown by Toro et al. [37] and modification of the microfiltration process as shown by Arndt et al. [38]. Eight diafiltration steps were carried out at constant filling level in order to transfer IgG and the other whey proteins in the microfiltration permeate to the feed tank of the ultrafiltration. To keep the natural milk milieu the protein-free permeate of the ultrafiltration unit was used as diafiltration media. After the first filtration process, a second filtration process was carried out only using the UF-plant and demineralized water as diafiltration liquid to decrease the salt and lactose concentration below 1%. The resulting native whey concentrates contained no

Table 1
Whey protein composition of native whey from milk and colostrum, data for isoelectric point (IEP) and molecular weight (MW) from [38].

Parameter	Colostral whey [%]	Milk whey [%]	IEP	MW [kDa]
α-La	1.79 ± 0.10	16.84 ± 1.3	4.2–4.5	14.2
LF	0.58 ± 0.05	2.16 ± 0.01	8.81	76.1
BSA	1.07 ± 0.03	3.11 ± 0.01	4.7–4.9	66.4
β-Lg	15.53 ± 0.27	63.07 ± 1.67	5.13	18.3
IgG	81.02 ± 0.45	14.81 ± 1.16	5.5–8.3	150–161

± Represents standard deviation of mean data from analytical quadruples.

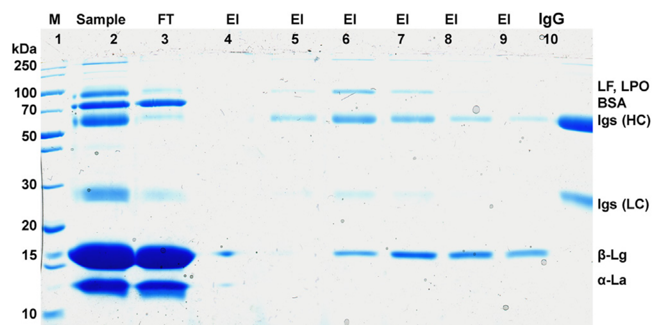

Fig. 2. Reducing SDS-PAGE (15%) of gradient elution. Lane 1: marker, lane 2: native whey sample, lane 3 MEP-flow-through, lanes 4–9 elution fractions at different pH, lane 10: IgG standard.

particles and hence were ideal for further chromatographic separation. Table 1 shows the whey protein composition of the obtained native whey from colostrum and milk.

2.2. Mixed mode resins for antibody purification

To separate IgG from other whey proteins two different mixed mode materials were used. The first was a Capto™- multimodal chromatography (MMC) material (GE Healthcare, Uppsala, Sweden) based on an agarose matrix. The average particle size of the beads is 75 μm and the dynamic binding capacity is 45 mg mL^{-1} BSA as given by the manufacturer. The Capto™-MMC has a ligand (N-benzoyl-homocysteine) with multimodal functionality. Due to its carboxyl group it is a weak cation exchanger but can also react based on ionic interactions, hydrogen bonding and hydrophobic interactions (see Data in Brief [39], Fig. 2). The process development was carried out using a prepacked HiTrap Capto™-MMC 1 mL column (GE Healthcare, Uppsala, Sweden). Upscaling of the chromatography process was performed in three steps (25 mL, 150 mL) up to a column volume of 3000 mL.

For direct capture of IgG the MEP HyperCel™ matrix (Pall Corporation, Port Washington, USA) was used. The material is composed of a porous cellulose matrix with abead-size between 80–100 μm linked with a 4-Mercapto-Ethyl-Pyridine (4-MEP) ligand. The given dynamic binding capacity was 20 mg mL^{-1} human IgG. With a pKa of 4.8, the MEP is uncharged at neutral pH so that the adsorption is mainly achieved by hydrophobic interactions (see [39], Fig. 1). The development of the purification method was carried out using a prepacked MEP HyperCel™ 1 mL column (Pall Corporation, Port Washington, USA). The corresponding column volumes for the three upscaling steps were 100 mL, 500 mL and 8800 mL.

2.3. Preparative purification of IgG from whey

The process development and the first two scaling steps were carried out on an ÄKTApurifier 100 UPC (GE Healthcare, Uppsala, Sweden) with a tunable flow rate up to 100 mL min^{-1}. The final upscaling step was done by using an ÄKTApilot (GE Healthcare, Uppsala, Sweden) system with a flow rate range from 4 to 400 mL min^{-1} for the MMC resin respectively with an Bio-Rad process chromatography station (Bio-Rad Laboratories GmbH, Munich, Germany) with a flow rate range from 83 to 2000 mL min^{-1} for the MEP column. The relevant operating parameters are summarized in Tables 2 and 3. The column volumes and the applied sample volume for MEP were scaled nearly linearly for the large scale production. The column volume of the MMC was reduced to one-quarter to one-third compared to the 1 mL MMC column during upscaling because of the different ratio of contained impurities to IgG when changing from milk whey to colostral whey.

For the process development, both mixed mode matrices were evaluated separately. During upscaling, both columns were coupled directly in the binding phase. The procedure for a run for both materials started with an equilibration phase of 5–10 bed volumes with the binding buffer (0.02 mol L^{-1} sodium phosphate (Merck KGaA, Darmstadt, Germany)/0.25 mol L^{-1} NaCl (Merck KGaA, Darmstadt, Germany) pH 7.5) until pH and conductivity were constant. The whey was adjusted to binding conditions and loaded to the column with a volume for which the amount of IgG did not exceed the maximum given binding capacity. The remaining sample was washed off with 5–10 bed volumes of the binding buffer until the baseline of UV absorption (280 nm). The columns were separated to start the elution process. The antibodies were eluted from the MEP-HyperCel™ by a stepwise manner with 0.050 mol L^{-1} MES (2- (N-morpholino) ethanesulfonic acid (Merck KGaA, Darmstadt, Germany) pH 6, 0.05 mol L^{-1} sodium acetate (Merck KGaA, Darmstadt, Germany) pH 4.5 and 0.1 mol L^{-1} Glycin/HCl (Carl Roth GmbH & Co. KG, Karlsruhe, Germany/VWR International GmbH, Darmstadt, Germany) pH 2.7 buffer. Both columns were regenerated with 0.5 mol L^{-1} NaOH (Germany/VWR International GmbH, Darmstadt, Germany). The elution buffer for the MMC column was 0.05 mol L^{-1} Glycin/NaOH (Carl Roth GmbH & Co. KG, Karlsruhe, Germany/VWR International GmbH, Darmstadt, Germany), 2 mol L^{-1} NaCl, pH 9. Flow-through and Elution fractions were collected for protein analysis.

2.4. Drying of purified IgG

The purified IgG was desalted below 5% of the initial salt concentration using a 10 kDa ultrafiltration polyethersulfone cassette membranes (Pall Corporation, Port Washington, USA), which retain the antibodies. The soluble salts were washed out from the concentrate (10 °C, 50.000 Pa transmembrane pressure) by the addition of demineralized water as diafiltration liquid. The lyophilization was carried out on a freeze dryer (ALPHA 1-4 LSC, Martin Christ GmbH, Osterode am Harz, Germany) over 48 h at 37 Pa, product area temperature of 15 °C and a condenser temperature of −52 °C.

2.5. Analytic of the isolation process by SDS-PAGE

The qualitative analysis with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli [40] with a 5% stacking and 15% separation gel. Samples and standards (IgG, Sigma Aldrich, St. Louis, USA) were diluted 1:4 with sample buffer and heated at 95 °C for 5 min. Protein separation occurred at 20 mA with a loading volume of 20 μL and bands were stained with Coomassie Brilliant Blue. Alternatively, TGX Stain-Free™ gradient gels 4–20% gradient gels were used according to Holzmüller and Kulozik [41]. Under reducing conditions the IgG molecule of ca. 150 kDa appears as two heavy chains (HC, 53 kDa) and two light chains (LC, 23 kDa) bands on the gel [42].

Table 2
Operating parameters for the MEP Hypercel™-column.

MEP volume [mL]	Sample volume[a] [mL]	Flow rate [mL min^{-1}]	Flow rate [cm h^{-1}]	Column diameter × bed height [cm]	Residence time [min]
1	0.25	0.2 (loading)	61	0.5 × 5	5
1		0.5 (elution)	153	0.5 × 5	
100	30	10	113	2.6 × 19	10
500	150	50	153	5 × 25.5	10
8800	3000	800	153	20 × 28	11

[a] Sample volume for colostral whey.

Table 3
Operating parameters for the Capto™- MMC-column.

MMC volume [mL]	Flow rate [mL min^{-1}]	Flow rate [cm h^{-1}]	Column diameter × bed height [cm]	Residence time [min]
1	0.2 (loading)	31	0.7 × 2.5	4.8
1	1 (elution)	156	0.7 × 2.5	
25	10	298	1.6 × 12.5	2.5
150	50	153	5 × 7.6	3
3000	400	306	10 × 38	7.5

2.6. Quantification of immunoglobulins and other whey proteins

The quantitative determination of IgG and the other whey proteins was done by reversed phase- high performance liquid chromatography (RP-HPLC) according to a method described by Voswinkel and Kulozik [43]. A PLRP-S 300 Å 8 μm column, 150 × 4.6 mm (Latek, Eppelheim, Germany) was used with an adapted elution gradient to increase the peak sharpness. The gradient after 15 min was increased over 6 min to 90% and the final increase to 100% was 30 s. For protein quantification samples were pre-diluted and the injection volume was varied between 20 and 80 μl to stay in the calibration range. Impurities by α-La, β-Lg, BSA and LF and LPO were detected without pre-dilution and the maximum injection volume. Additionally, IgG, IgA and IgM were determined using sandwich enzyme-linked immunosorbent assay (ELISA) test kits following the manufacturer's instructions (Bethyl Laboratories, USA, Cat. No. E10-118, E10-121 and E10-101). The yield of IgG (%) was calculated as the mass ratio of the bound IgG and IgG in the feed volume. The purity of IgG (%) was calculated as the ratio of IgG concentration eluted from the MEP column and the total whey protein respectively total protein concentration.

2.7. Total protein quantification

For total protein quantification, two different methods were used due to accuracy of the equivalent methods at high and low protein concentration. High total protein concentrations above 2% were measured according to the method from Dumas by determining the total nitrogen concentration using Nitrogen analyser vario MAX cube (Elementar Analysensysteme GmbH, Langenselbold, Germany). The nitrogen to protein conversion factor was 6.2 for pure IgG and 6.214 for colostral whey based on the protein composition given in Table 1 and the individual protein conversion factors [44]. Low total protein concentration (<2%) were measured using a Bicinchoninic Acid Protein (BCA) Assay Kit (product code BCA1, Sigma Aldrich, Germany) according to the manufacture's description. The assay and the photometric measurement at 562 nm are based on a method described by Lowry [45].

2.8. Analysis of the nativity of IgG with RP-HPLC

The degree of nativity was assessed by RP-HPLC using the same method as described above, whereas the activity was monitored by the ability to bind a specific antigen. The native state before and after the drying was also validated by differential scanning calorimetry (DSC), see below in chapter 2.10. To determine the degree of nativity the pH was adjusted to 4.6 with HCl. This leads to a precipitation of non-native molecules, which in the following were removed using a syringe filter of 0.45 μm (Chromafil RC-45/25 Macherey-Nagel, Dueren, Germany). The degree of nativity of IgG (%) was calculated as ratio of IgG concentration of the same sample with and without precipitation.

2.9. Analysis of the binding activity of IgG with ELISA

96-wells flat-bottom microtiter plates (Greiner, Austria) were coated with 5 μg mL^{-1} *Staphylococcus aureus* protein in 0.1 mol L^{-1} carbonate buffer (pH 9.0) (Merck KGaA, Darmstadt, Germany) After blocking of unspecific binding with 4% BSA (Serva, Germany) the plate was incubated with pre-diluted samples. Unbound material was washed off with PBS/Tween/1% BSA (Merck KGaA, Darmstadt, Germany). The detection antibody was anti-bovine IgG (H + L) conjugated with horseradish peroxidase (Jackson ImmunoResearch). The color reaction after the addition of tetramethybenzidine (TMB) (Sigma Aldrich, Germany) was stopped by adding H_2SO_4 (1N) and the optical density OD was measured at 450 nm with a Tecan Sunrise microtiter plate reader.

2.10. Analysis of the native state of IgG with DSC

The native state of the IgG powder after freeze-drying was verified by differential scanning calorimetry (DSC Q1000, TA Instruments, Alzenau, Germany). The powder was rehydrated to a concentration of 10 mg mL^{-1} IgG with simulated milk ultrafiltrate according to Dumpler et al. [46], of which, 20 mg were filled into a stainless steel pan. The heating rate was 2 C min^{-1} from 20 °C to 95 °C after an equilibration time of 2 min at 20 °C. The reference sample was a pan containing 20 mg of the solvent. The denaturation temperature was the maximum of the endothermic peaks and the enthalpy was calculated by integrating the equivalent areas.

3. Results and discussion

3.1. Direct capture of IgG from whey using a MEP Hypercel™ column

The chromatograms (Fig. 1) of the obtained native whey from colostrum (A) and milk (B) show all major and minor whey proteins. The binding mechanism to the RP-column is based on hydrophobic interactions and the elution from the column takes place by increasing the polarity of the mobile phase. This means that the later the whey protein elutes from the column, the more hydropho-

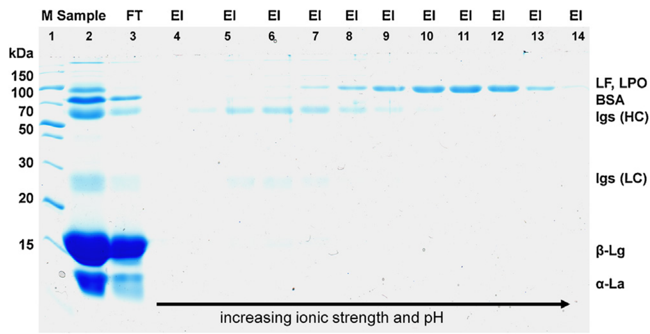

Fig. 3. Reducing SDS-PAGE (15%) during linear salt gradient elution of the Capto^{TM}-MMC column: lane 1 marker, lane 2 sample, lane 3 MMC-flow-through, lanes 4–14: elution fractions at different ionic strength and pH.

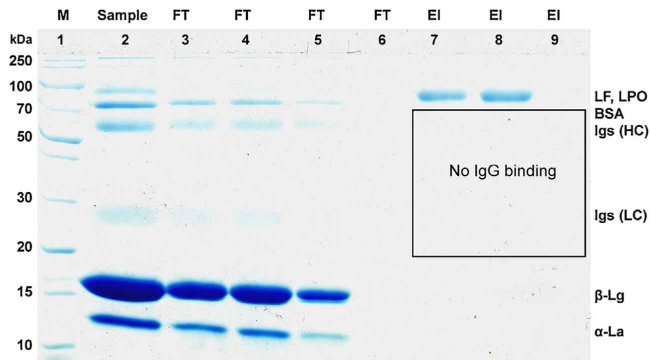

Fig. 4. Reducing SDS-PAGE (15%) during stepwise salt elution of the Capto^{TM}-MMC column with increase ionic strength during binding: lane 1 marker, lane 2 sample, lanes 3–6 MMC-flow-through, lanes 7–9: elution fractions at different ionic strength.

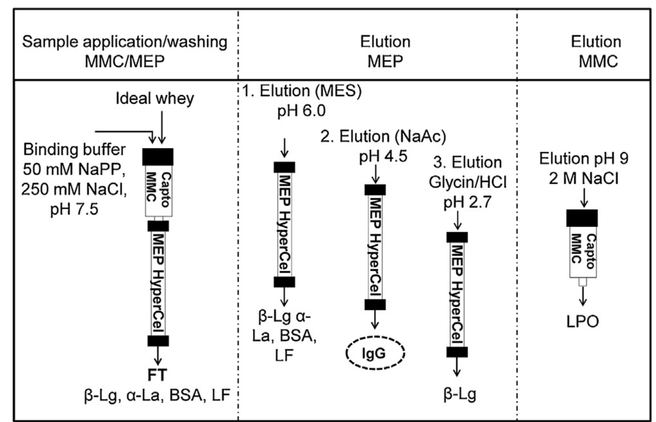

Fig. 5. Schematic description of the entire isolation process.

bic the character of the protein. Hence, the chromatograms show the hydrophobicity order of the whey proteins α-La < LF < BSA < β-Lg < LPO < IgG. Based on the RP-HPLC analysis we measured that IgG was more hydrophobic than any other whey protein. This property was used to develop a purification protocol for isolating pure IgG in a binding step using the mixed mode material MEP Hypercel^{TM}. The remaining whey proteins were in the flow-through. Fig. 2 shows a SDS-gel under reducing conditions of a pH/salt gradient elution from 0 to 100% using a 0.1 mol L^{-1} mM Glycin buffer at pH 2.7 over 10 bed volumes. By comparing the whey sample (Fig. 2, lane 2) and the flow through (lane 3) it can be seen that α-La, BSA, LF and most of the β-Lg are in the flow-through fraction. Impurities in the different elution fractions are β-Lg and LPO. LPO had to be identified by RP-HPLC (see Fig. 5D), because it cannot be differentiated with SDS-PAGE from LF due to the similar molecular weight. The flow-through and elution behavior is in accordance with the underlying binding mechanism (see [39], Fig. 1). At binding conditions at pH 7.5, hydrophobic interactions are dominant [47] so that the comparable hydrophilic molecules α-La, BSA, LF do not interact with the column and thus are found in the flow-through fraction, whereas the comparable hydrophobic proteins β-Lg and LPO show some binding. The optimal elution pH of 4.5 for IgG was determined dur-

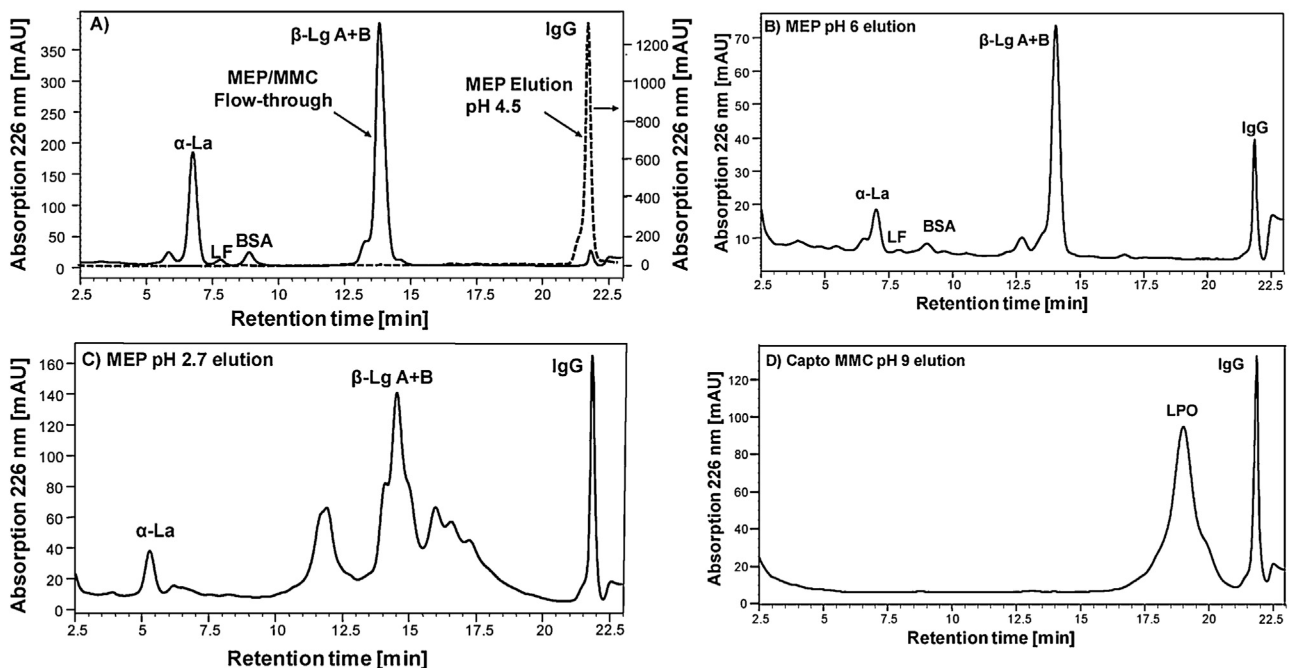

Fig. 6. RP-HPLC chromatograms of the isolation process: A: MMC/MEP flow-through (solid), MEP primary elution fraction at pH 4.5 (doted); B: MEP Elution at pH 6; C: MEP Elution at pH 2.7; D: Capto^{TM}-MMC Elution at pH 9 (because of different pre-dilution and injection volumes the comparison between the chromatograms is qualitative). For equivalent SDS-PAGE data see [39].

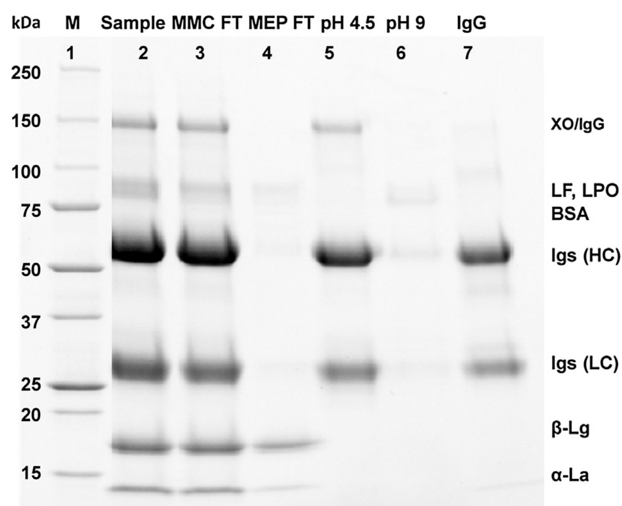

Fig. 7. Reducing SDS-PAGE during stepwise elution at highest scale (MMC/MEP: 3000 mL/8800 mL) using colostral whey: lane 1 marker, lane 2 sample, lane 3 MMC-flow-through, lane 4 MEP-flow-through, lane 5 MEP-elution at pH 4.5, lane 9 MMC elution at pH 9, lane 7 IgG standard. For equivalent RP-HPLC data see [39].

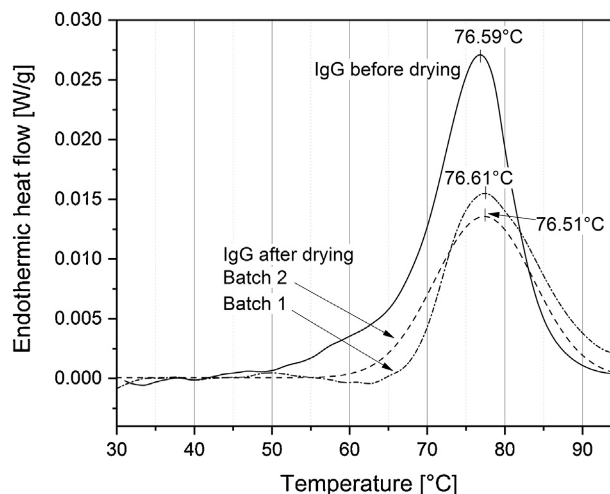

Fig. 8. DSC thermogram of IgG (10 mg mL^{-1}) in protein free simulated milk ultrafiltrate before and after drying, heating rate 2 C min^{-1}.

ing pH gradient elution ([39], Figs. 3–6) At this pH the pyridine ring of the MEP-ligand as well as IgG are positively charged, which leads to the electrostatic repulsion of the protein (see also chapter 3.3). The direct capture of IgG from whey has also the benefit of effectively removing microbiological contaminations naturally occurring in milk or colostrum during the manufacturing process.

3.2. Depletion of lactoperoxidase from whey using a Capto^{TM}-MMC column

Capto^{TM}-MMC as second mixed mode material was selected to remove impurities of LPO from the whey prior to the administration to the MEP column. For MMC the same binding conditions apply as for MEP Hypercel^{TM} (0.02 mol L^{-1} sodium phosphate pH 7.5), which allows a serial connection of both columns. At pH 7.5, the carboxyl group of the ligand is negatively charged ([39], Fig. 2). Hence, the positively charged LPO with an isoelectric point at pH 9.6 should bind to the column. Fig. 3 shows a SDS-gel with samples of a linear salt gradient elution with a 0.05 mol L^{-1} Tris/HCl pH 9 and 1 M NaCl buffer. It is obvious that both IgG and a band at 80 kDa were found in the different elution fractions (lane 4–14). The band at 80 kDa most probably represents the whey proteins LF and LPO, which are positively charged at this pH due to their high isoelectric point (IEP) at pH 8.8, respectively pH 9.6. Moreover, IgG elutes already at lower ionic strength and pH. This means that the undesired binding of IgG to the column should be based on electrostatic interactions that decrease with increasing ionic strength and pH. Due to the higher IEP of LF and LPO their interactions are stronger and, hence, they elute at higher ionic strengths and pH. As conclusion from these results, either an increase of the pH or an increase of the ionic strength during the binding phase should be applied to prevent binding of IgG to the column.

For this reason, the next step was to increase the pH from 7.5 to 9, which however, did not change the interaction of IgG with the column ([39], Fig. 7). A second step was to increase the ionic strength by addition of 0.25 mol L^{-1} NaCl during the binding phase (Fig. 4). To better detect impurities the elution with a higher ionic strength was carried out stepwise and with four times the sample volume. By increasing the ionic strength the electrostatic interactions are reduced in that way that IgG does not bind, whereas the interactions of LPO with an IEP of 9.6 and the column still takes place. Moreover, a band at 80 kDa was detected in the flow-through fraction (Fig. 4, lanes 3–5), which was not the case before the enhancement of the ionic strength (Fig. 3, lane 3). The expectation that this band represents Lactoferrin was validated at a later stage by the lack of Lactoferrin in the MMC elution fraction (Fig. 5D). Analogue to IgG, the electrostatic interactions of Lactoferrin are reduced by the addition of NaCl, thus found in the flow-through fraction.

The adapted binding conditions did not change the interactions between IgG and the MEP column, when applying the flow through of the MMC column to the MEP column ([39], Fig. 8). This is in agreement with Boschetti [48] as well as Wrzosek et al. [49], who showed that the adsorption capacity of monoclonal antibodies to the MEP column stayed constant at 41 mg mL^{-1} when increasing the NaCl concentration from 0 to 0.225 mol L^{-1}. The results were validated with bovine IgG by Shi et al. [50] with the justification that a higher ionic strength is beneficial for a faster mass transfer in the pores. Regarding pH, a lower pH of 6 [34], respectively higher pH of 9 [50], could slightly increase the adsorption of IgG to the MEP column. However, a pH of 6 would most probably induce interactions between IgG and the MMC column, whereas a pH of 9 would reduce interactions between LPO and the MMC resin. In consequence, both pH (6 and 9) for binding would result in a lower purity. The conclusion was that the implemented binding conditions (0.02 mol L^{-1} sodium phosphate, pH 7.5 and 0.25 mol L^{-1} NaCl) are suitable for the depletion of LPO from whey, while leading to only small losses in the IgG fraction. At the same time, the binding conditions lead to a high adsorption capacity of IgG to the MEP column [50].

3.3. Serial connection for IgG purification

The next step was the serial connection of both columns to prove the functionality of the complete purification process of IgG. A schematic description is given in Fig. 5 and the equivalent data in Fig. 6 . Related to the initial concentration in the whey 83.4% of α-La, 60.4% of LF, 90.42% of BSA, 84.5% of β-Lg and 12.2% of IgG were found in the MMC/MEP flow-through (Fig. 5A). After sample application and washing, the two columns were separated and the stepwise elution was done individually. Marginal impurities (below 1%), primarily caused by β-Lg, in elution fraction at pH 4.5 were removed by the introduction of another elution step at pH 6 (Fig. 5B, [39] Fig. 9). The pH reduction decreases the hydrophobic interactions and thus resulting in the desorption of the proteins for which the interactions are weak anyways. The IgG loss in this step was below 0.1%. By further reducing the pH to 4.5 both, the pyridine ring of the MEP-ligand and IgG are protonated thus positively charged. In

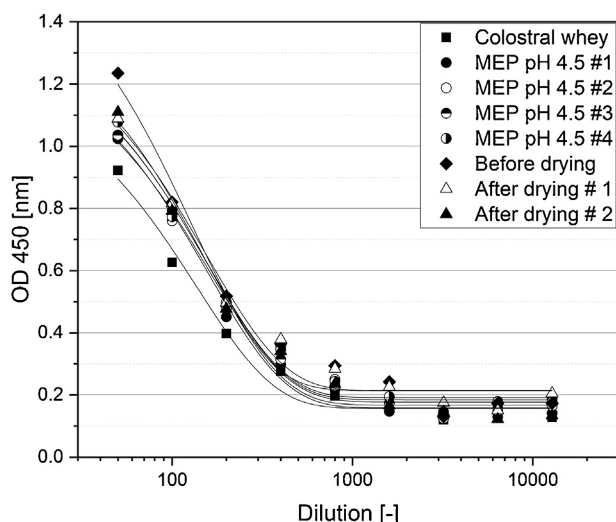

Fig. 9. Activity of IgG to bind *Staphylococcus aureus* protein during serial dilution at 10 mgmL^{-1} total IgG starting concentration.

Table 4
Purity and yield of IgG during upscaling.

MMC [mL]	MEP [mL]	Purity[a] [%]	Yield [%]
1	1	–	–
25	100	99.53	80.16
150[c]	500	99.84 ± 0.03	80.14 ± 1.65
8800[b]	3000	99.79 ± 0.03	66.2 ± 4.47

[a]Based on total whey protein, ± represents standard deviation of mean data from four, [b]Respectively five,[c]Individual cycles.

consequence, electrostatic repulsion leads to the desorption of the protein. No other whey proteins but IgG were found in the predominant elution fraction at pH 4.5, even when excessively overloading the RP-HPLC column (Fig. 5A elution). IgG with an IEP at pH 5.5–8.3 has a strong positive charge at pH 4.5, whereas the charge of β-Lg is weak positive (IEP at pH 5.13) and of α-La (IEP at pH 4.2–4.5) weak negative, thus only the reduction to pH 2.7 leads to the desorption of remaining bound whey proteins (Fig. 5C).

The elution of LPO and some IgG from the MMC was achieved by increasing the ionic strength using 2 M NaCl at pH 9. This in turn leads to a reduction of the electrostatic interaction and the desorption of the proteins. The lack of Lactoferrin in the MMC elution fraction is in agreement with the results shown in Figs. 3 and 4. The yield of IgG was 74.9%, with 12.2% loss in the flow-through, <0.1% at pH 6, 3.3% at pH 2.7 and 2.5% at pH 9. The remaining 6.7% were recovered in a side fraction of the pH 4.5 elution. The IgG losses in the flow-through fraction were eliminated by increasing the residence time from five to 10 min during upscaling.

3.4. Upscaling and reproducibility of the process

The process development was carried out with milk whey and colostral whey using prepacked 1 mL columns. Upscaling of the chromatography process was performed with colostral whey in three steps up to a column volume of 8800 mL for the MEP column and 3000 mL for the Capto^{TM}-MMC column. Table 4 shows the yield and purity during the different scaling steps. The purity was >99.5% based on the total whey protein content (Table 4) and >94% based on the total protein content (Table 5). The differences are due to small analytical inaccuracies. It is more imprecise to compare two different analytical methods with different principles and sample preparation than to compare two samples using the same method. At the highest scale MMC/MEP (3000 mL/8800 mL) it was possible to obtain 130–150 g pure IgG from 3 L of colostrum within 5 h, including the process steps sample application, washing, elution and regeneration of both columns ([39], Figs. 18, 20) Consistent data regarding purity and Ig content were found during four individual process cylces, showing a good reproducibility of the developed process ([39], Figs. 11–17).

Table 5
Purity and immunoglobulin composition of major elution fraction at pH 4.5 during four individual cycles at largest scale (3000/8800 mL).

Parameter	Batch 1	Batch 2	Batch 3	Batch 4
IgG (HPLC) [%]	1.38	1.16	1.12	1.62
Purity IgG [%][a]	98.31	95.02	94.11	96.78
IgG [%][b]	97.60	97.12	97.41	98.28
IgA [%][b]	1.42	1.59	1.52	1.06
IgM [%][b]	0.98	1.29	1.07	0.66
Total protein [%]	1.41	1.22	1.19	1.67

[a] Based on total protein, total protein measured with BCA.
[b] Relative immunoglobulin composition.

Fig. 7 shows the equivalent stained free SDS-PAGE of the isolation procedure at the highest scale. For equivalent RP-HPLC data see [39]. In analogy to the isolation from milk whey, it can be seen that the whey proteins α-La, β-Lg, IgG and LF are found in the flow-through fraction of the MMC (Fig. 6, lane 3) and α-La, β-Lg and LF in the flow-through fraction of the MEP column (lane 4). The lack of BSA on the gel is caused by two facts: its low concentration in the colostral whey (1.07%, Table 1) and the principle of the used stained free gel. The mechanism of the stained free gels is based on the reaction of trichlorethanol with tryptophan residues of proteins after the irradiation with UV-light. With only two tryptophan residues (0.56% relative to the molecular weight) according to results from Holzmüller and Kulozik [41], it is not possible to detect BSA in the sample or any other fraction. In comparison to the IgG standard (lane 7) a band at ca 150 kDa was detected, which is also observable on the SDS-gel shown by Wu et al. [35] One explanation could be that the band can be assigned to the milkfat globule protein xanthinoxidase (155 kDa) [51]. In order to collect larger amounts, colostrum is frozen at farm level. The freezing and defrosting before processing leads to a partial destruction of the milk fat globule membrane and hence a higher content of free proteins, which are attached to or integrated in the fat globule-membrane at native state. However, no other typical bands of milk fat globule proteins such as butyrophilin (66–67 kDa), periodic acid Schiff 3 (95–100 kDa) or lactadherin or periodic acid Schiff 6/7 (43–59 kDa) [51] were found in the sample (lane 2). Another explanation might be that the band represents non-reducible IgG. Another hint that the band could represent IgG is the purity of 98.31% based on the total protein content (Table 5, batch 1) whereas the relative intensity of the unknown band is 6.8% compared to the total intensity (lane 5). Hence, the purity should be lower if the band would not represent IgG.

3.5. Comparison between IgG and IgA and IgM

As it is difficult to differentiate between IgG and the other immunoglobulins, i.e. IgA and IgM, occurring in colostrum on an SDS-gel, the performance of all three immunoglobulins was additionally measured by ELISA (Table 6). Their relative concentration in the colostral whey was 86.94% IgG, 9.26% IgM and 3.80% IgA (Table 7). In contrast, the relative Ig composition in the main MEP-elution fraction at pH 4.5 was 97.6% IgG, 1% IgM and 1.4% IgA, which equals to 61.26% of the initial IgG, 15.99% of the initial IgA, 5.38% of the initial IgM. The yield for IgG is in good agreement with the data obtained from the RP-HPLC analysis (66.2%, Table 4). Compared to the smaller scales (yield >80%), the yield at highest scale was about 15% lower. This is because the focus laid on purity. Hence, the

Table 6
Relative immunoglobulin composition along the isolation process.

Parameter	Relative amount of IgG	Relative amount of IgA	Relative amount of IgM
Colostral whey	100	100	100
MMC FT	105.38 ± 15.882	77.82 ± 5.96	63.53 ± 6.01
MMC pH 9	2.38 ± 0.539	5.98 ± 1.265	4.63 ± 0.46
MEP FT	0.76 ± 0.425	8.21 ± 1.400	17.83 ± 4.150
MEP pH 6.0	0.12 ± 0.123	0.10 ± 0.069	0.164 ± 0.043
MEP pH 4.5	61.26 ± 7.703	15.99 ± 2.65	5.38 ± 1.082
MEP pH 2.7	0.03 ± 0.032	0.03 ± 0.017	0.07 ± 0.048

± Represents standard deviation of mean data from four individual cycles at the highest scale.

Table 7
Purity and nativity of obtained IgG powder.

Parameter	Colostral whey	Desalted liquid	Freeze dried batch 1	Freeze dried batch 2
IgG (HPLC) [%]	5.59	1.04	97.15	97.45
Purity IgG [%][a]	81.37	100[b]	100[b]	98.94
Nativity IgG (HPLC) [%]	98.47	94.52	96.89	95.17
IgG [%][c]	86.94	97.64	99.23	98.45
IgA [%][c]	3.80	1.39	0.41	0.66
IgM [%][c]	9.26	0.97	0.36	0.90
Total protein [%]	6.88	0.97	94.30	98.49
Dry matter [%]	NA	NA	96.3	96.32

[a] Based on total protein, total protein measured according to Dumas.
[b] Values were set to 100%, calculated values were slightly above 100% due to analytical inaccuracy.
[c] Relative immunoglobulin composition.

remaining material was recovered in side elution fractions. However, for the isolation process it is not the starting material, which is limiting, but the isolation process itself, thus this yield is considered acceptable. For IgA 77.82% were found in the flow-through of MMC column and 6% the elution fraction at pH 9, indicating some interaction with the MMC column. For the MEP column, 8.2% % were in the flow-through, but only 16.0% in the primary elution fraction, whereas the yield in the other fractions was negligible. Analogue as for IgA, IgM showed some interaction with the MMC column expressed by 36.5% fewer concentration in the flow through of the MMC resin. For the MEP 17.8% % were detected in the flow-through and only 5.4% in elution fraction. Even though unknown, it is expected that the hydrophobicity of bovine IgM and IgA should be similar to IgG due to the similar build up and structure. It should therefore bind to the MEP-column at pH 7.5. It has been reported that the binding between the MEP Hypercel™ column and IgG primarily takes place at the CH2 constant region of IgG [47]. Whereas for IgG the CH2 region is well accessible, the region might be inaccessible for sterical reasons for IgA, and even more for IgM. This might be an explanation for the unbound IgA and IgM in the flow-through. However, it is unclear what happens with the remaining IgA and IgM. An explanation might be that the primary elution of IgA and IgM takes place at a slightly lower pH, which was described for the human IgA and IgM [52]. Due to the lower isoelectric point of human IgA (pH 4.5–6.8) and IgM (pH 4.5–6.5) [53] compared to IgG the electrostatic repulsion is weaker at pH 4.5. Thus, the elution of IgA and IgM occurs only partially. Immunoglobulins start denaturing below pH 4 [54]. Therefore, they cannot be detected in the pH 2.7 fraction.

3.6. Effect of drying on isolated IgG

To preserve the isolated IgG for an extended period of time, the purified IgG fraction was desalted and freeze-dried, which had no effect on the particle size of IgG [39]. The stability of proteins is commonly investigated using DSC as analytical method. The measured denaturation temperature for IgG from two independent drying batches was found to be 76.61 °C and 76.51 °C and hence the same temperature as before drying 76.59 °C (Fig. 8). This is in the range of results for human IgG [55], which indicates the native state of the proteins. However, the denaturation enthalpy, which is the integral area underneath the curve resulting from the sum of unfolding reactions, was 0.149 J g^{-1}, respectively 0.142 J g^{-1}, compared to 0.457 J g^{-1} before freeze-drying. Additionally, the thermogram curve before drying starts to show upwards at 50 °C showing the start of protein unfolding, whereas the curves after the drying only starts to increase above 60 °C. This suggests that some unfolding already takes place during the drying process, which, however, has only little impact on the nativity and activity (see 3.7) of IgG. The nativity of the two batches was 96.89% respectively 95.17% (Table 7) showing that it is possible to produce native IgG in powdered form.

3.7. Impact of the isolation and drying process on the IgG activity

The objective of isolating bovine IgG is to use its physiological efficacy for various human health issues [56]. Thus, it is critical that the IgG retains its activity over the isolation process. As shown in Section 3.6 the nativity was >95%. It should be noted that the two values nativity and activity might not necessarily correspond. The nativity was measured with RP-HPLC and the activity with ELISA. The binding mechanism to the RP-column is based on hydrophobic interactions and hence the total concentration of IgG is measured and related to the total concentration after the precipitation of non-native molecules by adjusting the pH to 4.6. By this adjustment also partially denatured protein with deranged native conformation that may yet retain physiological functionality are precipitated and would hence not be measured. In contrast, ELISA measures the ability to bind a specific antigen. In our case, we studied the ability of IgG to bind *Staphylococcus aureus* protein by ELISA. In order to do so, the colostral whey and the MEP elution fractions were prediluted to equal concentrations (10 mg mL^{-1} total IgG based on the RP-HPLC measurement) and serial dilution was carried out (Fig. 9). By comparing the curves, it is obvious that neither the isolation process nor the drying had an effect on the activity.

4. Conclusion

The aim of this work was to develop a novel reproducible and scalable process to isolate IgG from colostrum with high purity and

recovery and minimal loss of activity. The results showed that the serial connection of two chromatography columns containing the mixed mode materials Capto^{TM}-MMC and MEP-Hypercel^{TM} lead to an average purity of 96.1% IgG based on the total protein content. The first column removed impurities of lactoperoxidase, whereas the second column was used for direct capture of IgG. The direct capture step has the major advantage that pathogenic microorganism, which occur naturally in a high amount in colostrum, are removed from the IgG. In conclusion, the obtained IgG products are virtually free of microorganisms. The binding took place under physiological conditions and elution at safe conditions for IgG (pH 4.5). By applying these comparatively mild conditions, it was possible to retain the activity and nativity (>95%) of IgG during the isolation process and during the downstream freeze-drying process. The process was developed at 1 mL scale and upscaling was done in three steps up to a column volume MMC/MEP of 3000/8800 mL. At the highest scale it was possible to yield 130–150 g pure IgG from 3 L of colostral whey within 5 h including the cleaning of the columns. The colostrum used in this study naturally contained antibodies against *Staphylococcus aureus*. This characteristic may be used to treat *Staphylococcus aureus* infection on the skin of patients. This specific activity of immunoglobulins in colostrum could be directed and increased against various pathogens via immunizing the cow. In conclusion, by applying the proposed isolation process, possibly in combination with immunizing the cow, it becomes feasible to obtain pure, active and stable IgG in therapeutic amounts. Thus, it becomes viable to generate novel and innovative IgG products for the treatment of microbiological infections in humans.

Even though the original aim was to develop an isolation process for colostral whey, the generated process is also highly suitable for the isolation of IgG from milk whey, which is available in unlimited amounts and less complicated to collect than colostrum. All major whey proteins (β-Lg, α-La, BSA) were found in the flow-through fraction and, hence, they do not reduce the binding capacity. This opens the possibility to cost-efficiently obtain large amounts of IgG from milk whey.

Conflict of interest

The authors claim no conflict of interest.

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