Separation of Immunoglobulins from Colostrum using Methods Based on Salting-out Techniques

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Abstract

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This study aimed to obtain a purified IgG fraction from bovine colostrum using salting-out precipitation methods as base techniques. As the first step after skimming of colostrum, isoelectric precipitation or rennet coagulation were used for casein removal. IgG concentrations in raw material and final products were determined using the ELISA method. SDS-PAGE electrophoresis was used for purity determination of the final fraction of IgG. According to the obtained results, the combination of precipitation, gel filtration, and cross-flow filtration techniques enables to separate the IgG fraction with purity up to 90% and yield up to 91%. Acid whey has a higher yield in comparison with sweet whey and both ammonium sulphate and sodium sulphate have similar effectivity for precipitation of IgG. The final concentrate of IgG can be used as a standalone product or as an additive to other food supplements.

Keywords: ammonium sulphate; cross-flow filtration; gel filtration; sodium sulphate

Immunoglobulins (Igs) are important animal glycoproteins. The main function of Igs is the protection of the organism against infection. These biomolecules have a molecular weight in the range of 146–900 kDa (Holliger & Hudson 2005; Lonberg 2005). From the commercial point of view, Igs are the base for pharmaceuticals, diagnostic, and laboratory test kits, recently also for food supplements. Bovine colostrum (BC) is a raw material which is one of the most saturated by IgG among natural IgG sources.

BC is a product secreted by the mammary glands of female mammals to nourish their young for a period beginning immediately after parturition. BC is available in quite large quantities and therefore is suitable for further processing. BC is vitally important for newborn calves due to its nutritional and protective functions. The protective function is ensured by the presence of a complex mixture of bioactive and antimicrobial proteins like IgG, lactoferrin, lactoperoxidase, lysozyme, and proline-rich polypeptides (Séverin & Wenshui 2005). The composition of BC changes rapidly after parturition. The concentration of IgG in BC can be over 50 g/l (Godden 2008; Vetter et al. 2013), while the concentration of IgG in cow's blood serum varies from 2.6 to 38 g/l (Gelsinger et al. 2015) and in mature bovine milk its content is in the range of 0.6 g/l to 1 g/l (O'Mahony & Fox 2013).

Protein A affinity chromatography is one of the most effective and common methods for separation of Igs in biotechnology. However, this technique has two disadvantages: low binding capacity (about 20–40 g IgG/l of particles (Ishihara et al. 2010)) and high

price (protein A is the most expensive among all industrial chromatography media (Gottshalk *et al.* 2012)). Ion exchange chromatography is commonly used for IgG capturing (Nian & Gagnon 2016), but chromatographic methods usually require expensive infrastructure which cannot be afforded by most of the dairy companies.

One of the oldest and in the same time effective IgG separation techniques are salting out precipitation methods, which are based on different protein solubility in the presence of ions. At a high salt concentration (> 0.5 mol/l), protein solubility decreases as ionic strength increases in the process known as 'salting out'. The salting-out ability of anions and salting-in ability of anions follows the Hofmeister series: increasing precipitation from thiocyanate cation (PO_4^{3-} > SO_4^{2-} > CH_3COO^- > Cl^- > B^- > ClO_4^- > I^- > SCN^-) and increasing chaotropic effect from ammonium cation (NH_4^+ > Rb^+ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+} > Ba^{2+}) (Duong-Ly & Gabelli 2014). One of the first observations of IgG separation by ammonium sulphate precipitation was published at the end of the 19th century (Ehrlich & Brieger 1893). During the next 70 years there were a lot of publications about separation of Igs. For separation of IgG from bovine BC, ammonium sulphate (AS) and zinc sulphate precipitation (Butler *et al.* 1972) was used, but only for laboratory purposes without yield analysis. Later it was published that precipitation of IgG is caused by adding appropriate amounts of salts, such as AS or sodium sulphate (SS) to all mammal's serum, plasma, ascites fluid, and hybridoma culture supernatant (Heide & Schwick 1978). Today AS and rarely SS are used for precipitation of IgG from different raw materials, but in some cases SS gives a purer product (Page & thorpe 2009). These salting-out methods have several critical advantages: they are cost effective and simple to process, there is a minimum of protein denaturation. That is why nowadays these methods are commonly used for the separation of monoclonal and polyclonal Igs in biotechnology (Page & thorpe 2009). Both AS (E517) and SS (E514) can be used as food additives.

In this study, we explored the different methods of IgG separation based on salting-out precipitation techniques. The main goal was to find out optimal conditions for the maximum yield of IgG separation with the potential for industrial scale up. Two types of IgG precipitation by salting out with AS and SS were used. Gel filtration (GF) and cross-flow filtration were used as intermediate and final steps.

MATERIAL AND METHODS

Samples. Samples of mixed BC were obtained from Ingredia LLC (Czech Republic). Altogether 2 l raw BCs were divided into 50 ml portions and frozen at –72°C.

Reagents. Trisma base, tetramethylethylenediamine, and ammonium persulfate and dithiothreitol were supplied by Sigma-Aldrich (Germany); SS, AS, sodium acetate, acetic acid 99%, sodium dodecyl sulphate, ethylenediaminetetraacetic acid disodium salt dihydrate, sodium phosphate monobasic, sodium phosphate dibasic, sulfuric acid, and glycerol were supplied by Lach-Ner (Czech Republic); 40% acrylamide/bis-acrylamide solution 19 : 1 was purchased from Bio-Rad (USA). The 0.22 μm nitrate cellulose membrane fixed in a Nalgene DS0320-5033 reusable bottle top filter holder (Thermo Fisher Scientific, USA) was used for filtration of all solutions and buffers.

Skimming of BC. Samples were thawed in a water bath at 4°C and centrifuged at 9000 *g* for 30 min at 4°C using a laboratory centrifuge Hettich UNIVERSAL 320 R (Germany). The supernatant (fat) was removed and skimmed BC was used for further experiments.

Preparation of acid whey (AW) from BC. Skimmed BC was 5 times diluted with deionised water before casein precipitation. Caseins in BC were precipitated at pH 4.6 with 0.5 mol/l HCl; pH was monitored with an Accumet AR25 pH meter (Thermo Fisher Scientific, USA). Acidified BC was mixed at 25°C for 30 min on the magnetic stirrer at 270/minute. Thereafter, AW was separated by centrifugation at 4200 *g* for 30 min at 25°C using the centrifuge mentioned above.

Sweet whey (SW) preparation from BC. Twice diluted BC was used for this processing. Caseins in BC were coagulated with rennet (Naturen; Christian Hansen, Denmark) on a magnetic stirrer at 270/min and 35°C. For 1 kg of colostrum was used 0.5 ml of rennet. SW was separated by centrifugation using the same conditions as in AW.

Salting-out with ammonium sulphate and sodium sulphate. Dry AS or SS were added gently to get 11 different final proportions of salting-out agent from 15 g to 25 g/100 g of whey with 1 g/step. The mixture was placed on the magnetic stirrer for 1 h at 25°C. Precipitated proteins were separated by centrifugation at 9000 *g* for 15 min at 25°C. The supernatant was discarded, and pellets were washed with the solution of the same concentration of AS or SS, which was used for precipitation. After washing, centrifugation was repeated under the same condi-

tions. Final pellets were dissolved in 50 mmol/l phosphate buffer (pH 6.5). Pellets which were obtained from 100 ml of AW and SW were diluted in 20 and 50 ml of buffer, respectively. Obtained fractions of proteins were used for further processing and analysis.

Cross-flow filtration. Kvick Start cassette with 100 kDa cut-off and Start AXH cartridge with 0.65 μm pore size installed in an AKTA Flux S unit (GE Healthcare Life Sciences, Sweden) were used for ultrafiltration (UF) and microfiltration (MF). The constant transmembrane pressure of 0.15 and 0.08 MPa at 25°C was used for UF and MF, respectively.

Gel filtration. Sephadex G50 Fine was packed in an XK26/10 column (bed dimensions 2.6 × 7.5 cm). The maximum flow rate was 7.5 ml/min (84.74 cm/h). All chromatographic processes were conducted using an AKTA Pure 25 system with the Unicorn 7.0 software. 50 mmol/l phosphate buffer (pH 6.5) was used as the mobile phase. All needed equipment and material for GF were supplied by GE Healthcare Life Sciences (Sweden).

Quantification of IgG1. For the quantification of IgG1 concentration in BC and fractions after BC processing the bovine IgG ELISA quantitation set (E10-118; Bethyl Laboratories, USA) was used. The used ELISA procedure was adapted from the instructions and the protocol described by VETTER *et al.* (2013). Samples of BC and BC fractions for this assay were diluted to get a final concentration of IgG1 in the range between 100 and 500 ng/g. Absorbance was measured using a PowerWave XS microplate reader (BioTek, USA) at the wavelength of 450 nm. Processing of obtained data was done by the online application www.elisaanalysis.com.

Polyacrylamide gel electrophoresis. The purity of separated IgG fractions was analysed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Stacking gel (4%) and separating gel (8%) (LAEMMLI 1970) under reducing conditions with dithiothreitol were used for electrophoretic separation. Gel casting and electrophoretic separation were done using Mini-PROTEAN Tetra Handcast
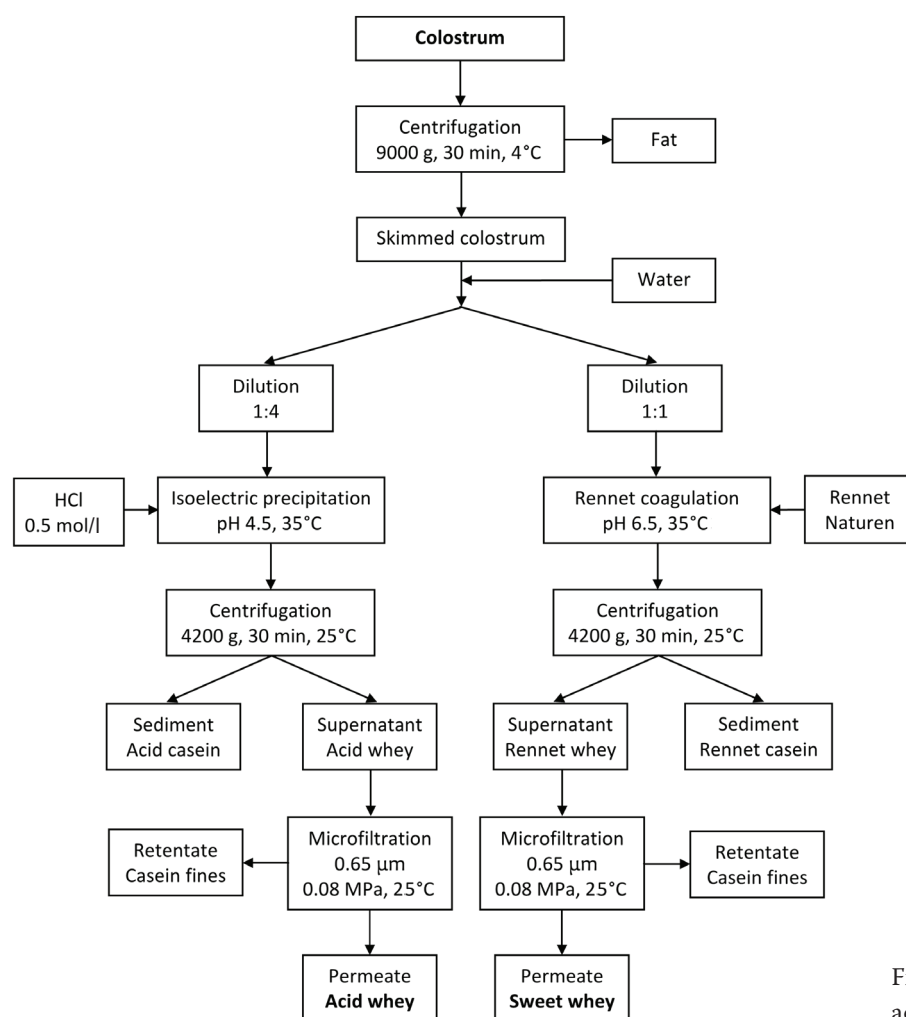

Figure 1. Preparation of sweet and acid whey from bovine colostrum

Systems (Bio-Rad, USA). QC Colloidal Coomassie (Bio-Rad, USA) was used for the staining of separated proteins. CLIQS software (Totallab, UK) was used for the processing of electrophoretograms.

RESULTS AND DISCUSSION

BC contains a high amount of fat, which can be up to 9% (Séverin & Wenshui 2005). We found that separation and clarification of the whey after casein precipitation were easier if BC was skimmed before processing. Fat from BC was removed by centrifugation at 9000 *g*, which was similar to conditions for industrial centrifugation in centrifugal separators (Bylund 2003). The concentration of IgG1 in the sample of skimmed BC estimated by ELISA assay was 58.4 ± 0.9 mg/g.

The next step was removal of caseins from skimmed BC by acid or rennet coagulation. Before these precipitations it was necessary to dilute BC. We have noticed that casein precipitation does not occur at pH 4.5–4.6 without dilution. An optimal dilution factor for isoelectric precipitation was 5. If the concentration of IgG1 was above 80 mg/g, it was necessary to dilute skimmed BC at least 8 times. Dilution factor 2 was used for rennet coagulation of casein, if the concentration of IgG1 was above 80 mg/g, suitable dilution factor was 4. However, it was observed with higher dilution factor that the coagulation of casein is slower and takes over 2 h to get the final transparent SW. The advantage of dilution procedure is also a decrease in the amount of the entrapped IgG in casein coagulum, which increases total yield of IgG. Piot *et al.* (2004) diluted colostrum before fat separation to reduce the loss of Igs in cream. It is advantageous if the centrifuge capacity is sufficient. Coagulation of casein and separation of AW and SW can easily be scaled up to a pilot and industrial scale including the centrifugation step, which can be done by using a decanter centrifuge. General steps of casein removal from colostrum are presented in Figure 1.

Obtained AW and SW were filtrated through a 0.65 μm membrane. This step removes all small particles of casein and improves the microbial quality of whey. The flow rate of permeate for SW was lower than that for AW (Figure 2), possibly it caused different dilution and as a result, different viscosity. The average flow rates of AW and SW were 260 and 191 kg/h/m^2, respectively. Piot *et al.* (2004) reported the flow rate about 10 times lower, but they used
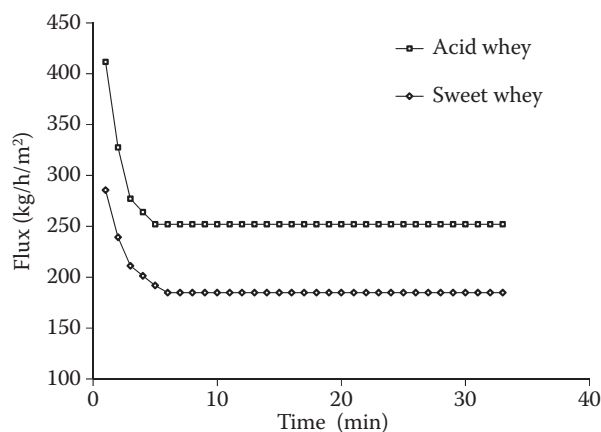

Figure 2. The flux of the permeate during the microfiltration of acid and sweet whey using a 0.65 μm cut-off membrane

a lower dilution and the membrane with pore size 0.1 μm. In our case, each litre of BC can be used for the production of 4.82 ± 0.02 kg of AW or 1.80 ± 0.02 kg of SW. This step of BC processing can also be scaled up easily. After filtration, step concentrations of IgG1 in AW and SW were 11.0 ± 0.7 and 28.0 ± 0.9 mg/g. It means that the yield of the coagulation step is about 90.9% for AW and 86.3% for SW.

Salting out and precipitation of Igs can be carried out directly without further concentration, but in this case a higher amount of reagent has to be used. SW and AW were 2 and 5 times concentrated, respectively, using the 100 kDa UF membrane (Figure 3) for a reduction of the material cost. In both cases,
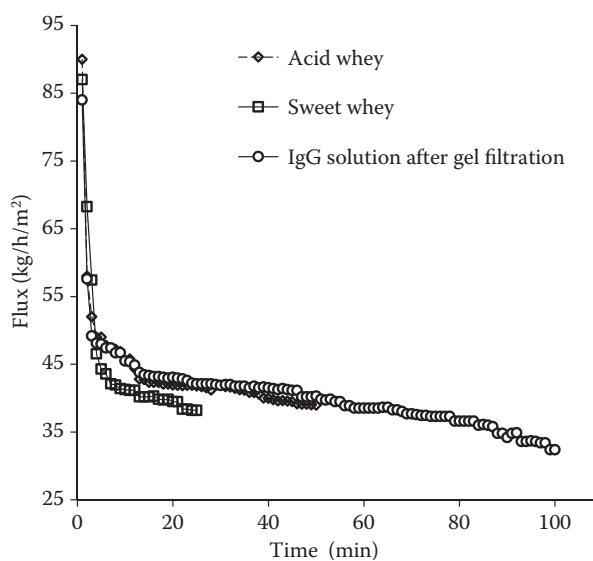

Figure 3. The flux of the permeate during the ultrafiltration of acid and sweet whey before salting out and during the ultrafiltration of IgG solution after gel filtration using a 100 kDa cut-off membrane
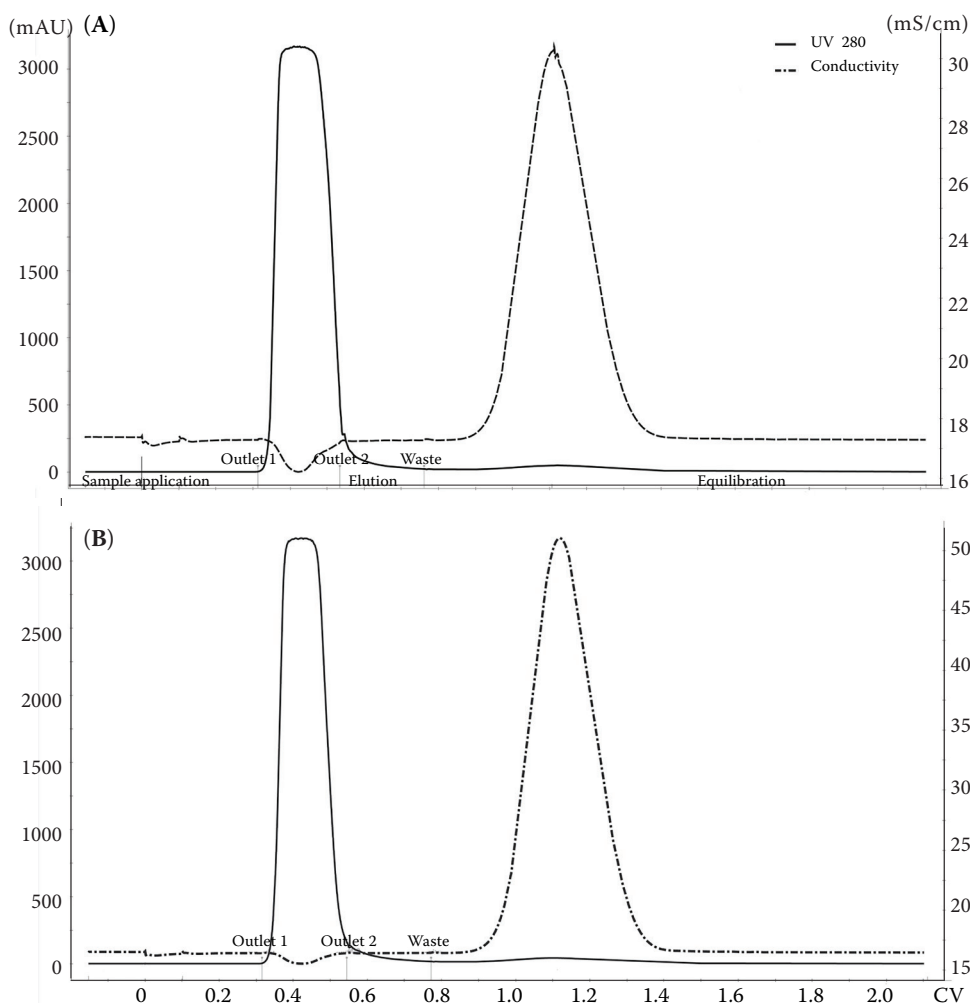
Figure 4. Chromatogram of the IgG fraction obtained after (**A**) sodium sulphate and (**B**) ammonium sulphate salting out

CV – column volumes

average permeate fluxes for AW and SW were quite similar 43.6 and 44.6 kg/h/m^2, respectively. However, if we compare the concentration of SW and AW obtained from the same amount of BC, in the case of the SW process of concentration takes twice less time compared to the time of AW concentration with the same volume. Concentrated whey was used for the precipitation of Igs with SS and AS.

The next step was to choose the optimal amount of reagents for salting out. The range of concentrations based on information in literature (PAGE & THORPE 2009) was selected. There is always a compromise between purity and yield. However, our aim was the maximal yield. We used therefore a different amount of precipitant to find the optimal conditions for either of the two reagents. Before quantification of IgG1 in the obtained fraction, it was necessary to dissolve pellets and to remove the precipitation agent. Pellets were dissolved by mixing on a magnetic stirrer in phosphate buffer pH 6.5. There was not observed any

Table 1. Yield (%) of immunoglobulin separation based on salting out techniques

Whey type	Salting out agent	Salting out agent (g/100 g of whey)										
		15	16	17	18	19	20	21	22	23	24	25
AW	AS	68.3 ±0.8	70.9 ±0.7	73.8 ±0.7	76.4 ±0.8	79.1 ±0.9	82.3 ±0.9	85.8 ±0.9	88.2 ±0.7	91.2 ±0.9	91.4 ±0.8	91.3 ±0.9
	SS	67.4 ±0.9	71.9 ±0.8	72.8 ±0.8	76.2 ±0.9	78.1 ±0.9	83.2 ±0.9	85.4 ±0.8	86.8 ±0.6	89.1 ±0.9	89.8 ±0.9	91.2 ±0.7
SW	AS	67.2 ±0.4	70.2 ±0.5	73.3 ±0.8	74.6 ±0.9	77.8 ±0.7	80.9 ±0.4	84.9 ±0.7	85.3 ±0.6	86.1 ±0.8	86.4 ±0.9	86.1 ±0.7
	SS	65.4 ±0.8	68.1 ±0.8	71.9 ±0.4	73.9 ±0.9	78.2 ±0.9	81.4 ±0.7	85.3 ±0.4	84.9 ±0.9	86.3 ±0.5	86.4 ±0.4	86.4 ±0.9

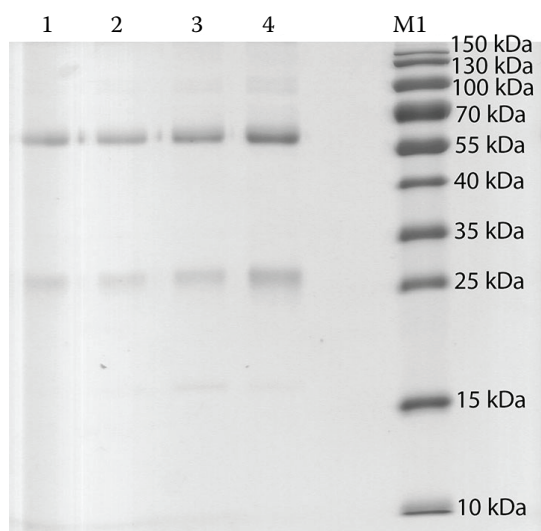AW – acid whey; SW – sweet whey; AS – ammonium sulphate; SS – sodium sulphate

Figure 5. Electrophoretogram of immunoglobulin fractions obtained by techniques based on different salting-out methods

1 – IgG fraction obtained by precipitation with sodium sulphate (SS) from acid whey (AW); 2 – IgG fraction obtained by precipitation with ammonium sulphate (AS) from AW; 3 – IgG fraction obtained by precipitation with SS from sweet whey (SW); 4 – IgG fraction obtained by precipitation with AS from SW; M1 – broad range molecular weight marker

difference in the dissolution time of precipitate obtained using SS or AS and this step took 15–17 minutes. The precipitate was dissolved in a similar volume of buffer which corresponded to the volume of BC before casein precipitation or coagulation.
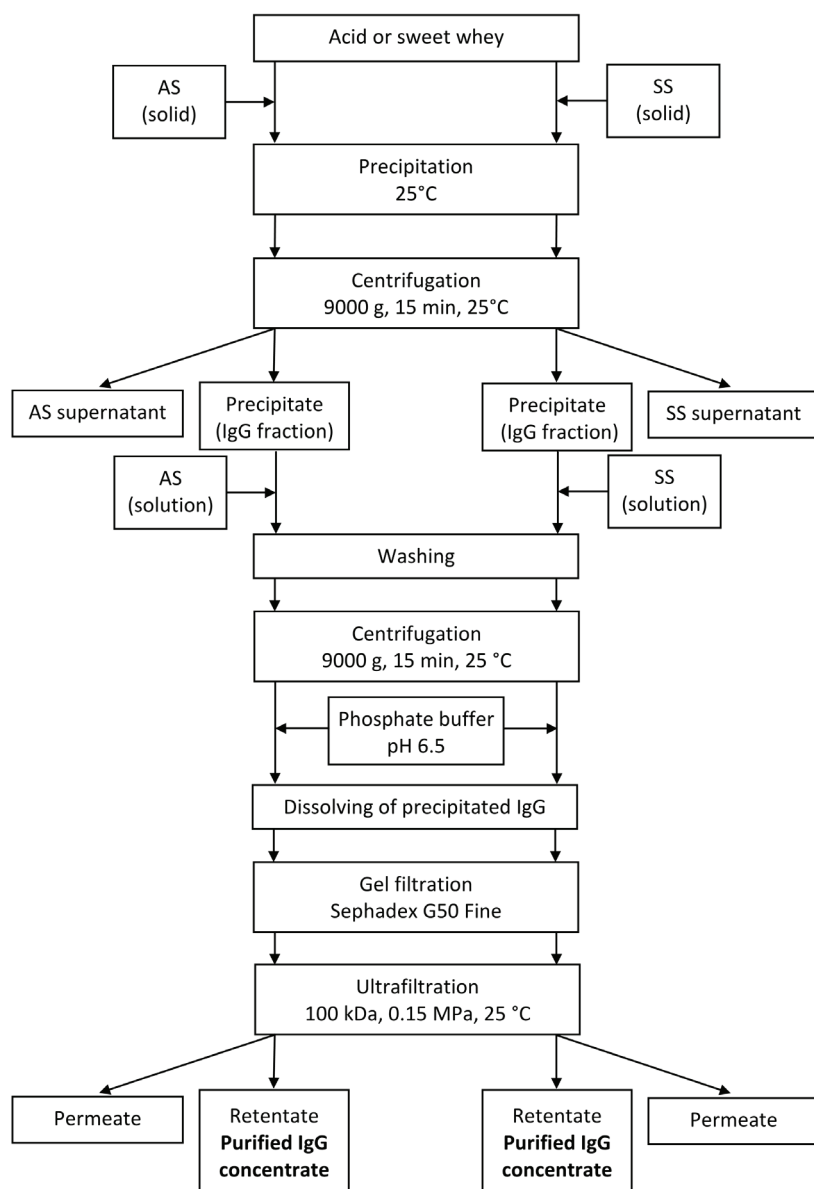

Figure 6. Schematic diagram of immunoglobulin separation based on salting-out techniques with ammonium sulphate (AS) and sodium sulphate (SS)

The Sephadex G50 fine column was used for removal of precipitant from the obtained fraction. This GF medium has an exclusion limit of 30 kDa. This feature is not critical for desalting but it could increase the separation of low molecular compounds. Additionally, the automatic column pressure control was used, which in a certain period of separation decreased the flow rate of the mobile phase to protect the column from damage by overpressure. Excellent separation of proteins is shown on chromatograms (Figure 4), which corresponds to an absorption signal at 280 nm, and salt, which corresponds to a signal from the conductometer. Additionally, there was observed a low absorption at 280 nm at the same retention time as the conductivity peak has. It could be some low-molecular-weight peptides or other biomolecules which can absorb light at 280 nm. Desalting or removing of the precipitation agent from 10.5 ml of solutions with Igs after salting out took about 9 min in our case. The dilution factor was about 1.33 for desalting by GF. Generally, 1 l of Sephadex G50 Fine can be used for high-performance removal of the precipitation agent with effectivity up to 1.5 kg of IgG fraction/1 h and with the consumption of about 9.6 l of buffer. On an industrial scale this parameter can be enhanced by choosing a coarser GF medium, like Sephadex G50 Coarse. GF can be replaced by UF combined with diafiltration, but in this case for removal of the precipitation agent from the IgG fraction at least 35% more buffer would be used. The permeate flux during UF varies from 29 to 45 kg/h/m^2. Salts can also be removed by electrodialysis followed by UF for scale up.

The fractions obtained after GF were concentrated by UF using 100 kDa cut-off membrane and volume concentration factor up to 10, average flux was 40.5 kg/h/m^2 (Figure 3).

Samples of the retentate after UF were analysed by ELISA to calculate the final yield of the entire process (Table 1). Yield of processing grows at a high concentration of the precipitant. Doses higher than 23 g/100 g of whey have only a small effect on yield regardless of the type of whey or precipitant. SDS-PAGE (Figure 5) has shown that the salting out of Igs from AW and SW can give purity about 90 ± 0.7 and 87 ± 0.7%, respectively, at a maximum yield. General steps of IgG separation from BC whey are described in Figure 6. Final concentration of IgG1 in the UF retentate was up to 170 mg/g.

Generally, AS and SS have the same effect and require the same amount of the precipitation agent. But AS

has higher solubility (76.4 g/100 g H$_2$O at 25°C), that is why this precipitation agent is regularly used in diluted form (Page & Thorpe 2009). Moreover, AS can be recovered from a supernatant using electrodialysis (Koter *et al.* 2015). SS has lover solubility (28.1 g/100 g H$_2$O at 25°C), therefore it can be used for processing only in dry form. SS can also be removed from the supernatant by electrodialysis (Kroupa *et al.* 2014), but the electrodialysis concentrate containing SS has to be dried before the next use. It was also observed that in SS precipitation, SS can be partially obtained from the supernatant by crystallisation after cooling down of the supernatant, solubility of SS is 5.0 g/100 g H$_2$O at 0°C. This feature of SS precipitation can be useful before electrodialysis. The final choice of whey type and precipitation agent for IgG separation will depend on prices of reagent and equipment which can be used.

CONCLUSION

Skimmed BC is a great raw material for IgG separation. The first step of BC processing after fat separation is always casein removal, which can be done either by rennet coagulation or isoelectric precipitation. In both cases BC has to be diluted twice and 5 times with water for rennet coagulation and isoelectric precipitation, respectively. This step increases the yield of IgG content in BC whey. Both types of whey can be processed directly by salting out, but UF is recommended to reduce the amount of the used precipitant. For all types of precipitation processes it is necessary to use GF, which can be scaled up to an industrial scale or replaced by membrane processes. The use of AW has a higher yield than that of SW, and both AS and SS have similar effectivity for the precipitation of IgG from BC whey. The combination of AW and AS precipitation can give a maximum yield plus AS can also be recycled for future use. Further advantage of the precipitation technique is a reduction of lactose concentration and other non-protein low molecular weight component in the final product. The final product which contained a high concentration of Igs can be used for enhancement and standardisation of BC quality or as a food supplement.

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