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Dairy wastewater utilization: separation of whey proteins in membrane and chromatographic processes

Magdalena Lech, Anita Niesobska, Anna Trusek-Holownia*

Department of Chemistry, Wroclaw University of Technology, Wybrzeze Wyspianskiego 27, 50-370 Wroclaw, Poland, emails: magdalena.lech@pwr.edu.pl (M. Lech), anita.niesobska@pwr.edu.pl (A. Niesobska), Tel. +48 71 320 2653; Fax: +48 71 320 2534; email: anna.trusek-holownia@pwr.edu.pl (A. Trusek-Holownia)

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ABSTRACT

Dairies are obligated to utilize whey after cheese production. From an environmental protection point of view, the high content of lactose and proteins in post-production wastes, like whey, is harmful for the environment. From another point of view, whey is a source of very valuable, active proteins, particularly lactoferrin and serum albumin. Their modulatory potential is exhibited in their pure form and improves after partial, controlled hydrolysis. Unfortunately, the fractionation of this multicomponent medium is not an easy task. The paper describes an integrated process of fractionation of whey proteins. After the first step of treatment based on membrane techniques, the concentrated, most valuable whey proteins were subjected to a few steps of chromatographic separation. The separation properties of the ultrafiltration membranes were unexpected. The typical cut-off boundary was shifted in the direction of components having a lower molecular weight. After laboratory-scale testing, a concept for an industrial-scale process for the isolation of the most valuable whey proteins with purities of nearly 100% was elaborated.

Keywords: Whey utilization; Dairy industry; Protein fractionation; Ultrafiltration; Affinity chromatography; Ion-exchange chromatography

1. Introduction

Dairies are obligated to utilize whey after cheese production. From an environmental protection point of view, the high content of lactose and proteins in post-production streams is harmful for the environment. These proteins could cause an imbalance in the biological life in water reservoirs. Therefore, the whey is subjected, *inter alia*, to biodegradation. The process may be accompanied by the release of biogas [1].

Simultaneously, the content of essential proteins makes whey a valuable product. Whey proteins include immunoglobulins, α-lactalbumin, β-lactoglobulin, serum albumin, lactoferrin, and lactoperoxidase [2]. Particularly, due to the physicochemical and functional properties of lactoferrin, methods for its separation are a subject of intensive research [3,4]. Lactoferrin exhibits immunomodulatory, antimicrobial,

*Corresponding author.

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antioxidant, anticarcinogenic, and anti-inflammatory properties. It has a high affinity for ions and transports and binds mainly Fe^{3+} ions [5,6]. Another particularly interesting protein is serum albumin. It functions as an antioxidant by protecting lipids from oxidation [7]. Albumin also has gelling properties that make this protein an object of interest in the food and pharmaceutical industries [8].

Fractionation of the mixture of proteins present in whey to pure fractions of the individual proteins can allow those with special nutritional needs to adjust their diet to improve their health. In particular, hydrolysates with great health benefits can be obtained by enzymatic hydrolysis of the individual compound [9,10]. The hydrolysates are better assimilated by the body than the protein, and their shorter sequences have even higher modulating properties than the initial protein [11,12].

Whey is a difficult medium for fractionation due to the diversity of the ingredients and their low concentrations. Whey proteins have been concentrated by membrane filtration, evaporation and spray drying to form whey protein concentrate [13]. The whey proteins differ in their molecular weights and isoelectric points (Table 1) [14]. The described processes for the production of fractionated whey proteins can be divided into four categories: selective precipitation [15,16], membrane separation [14,17–19], selective adsorption [20,21], and selective elution [3,22–25]. None of these solutions guarantee a comprehensive separation.

Based on the literature and our results, we aimed to integrate membrane filtration, gel filtration, and ion-exchange methods in order to obtain the particular fraction of the most important whey proteins. In the first step, we concentrated whey proteins by membrane filtration to obtain the most valuable proteins in the retentate. Then, we used a protocol [4] to isolate lactoferrin from goat colostrum. The separation of serum albumin from cow whey was described by Gerberding and Byers [22]. The authors applied anion-exchange chromatography on Q-Sepharose Big Beads. We used that approach to isolate serum albumin from goat whey but changed the resin used for DEAE-Sepharose and the elution phase for sodium chloride and added one more step of separation—gel filtration chromatography on Sephadex G-50.

2. Materials and methods

2.1. Materials

The goat whey was obtained from Kozio-Lek (Poland).

Membranes: polyethersulfone membrane **(**Poly-Mem, Poland), cut-off coefficient of 80 kD, membrane length—180 mm, number of capillaries—90, internal surface area—0.036 m^2. Ceramic membrane (Filtanium, distributed by Intermasz, Poland), cut-off coefficient of 150 kD, membrane length—250 mm, one tube with seven channels, internal surface area—0.013 m^2.

Chromatography resins, CM-Sepharose®, DEAE-Sepharose®, and Sephadex® G-50, were obtained from Sigma-Aldrich (Germany).

Lowry reagent, Folin-Ciocalteau Reagent, acrylamide, acrylamide/bisacrylamide, bromophenol blue sodium salt, ammonium persulfate (APS), N,N,N´,N´-tetramethylethylenediamine (TEMED), a Gel Filtration Marker Kit for protein molecular weights 6,500–66,000 Da, Trizma® base, sodium dodecyl sulphate (SDS), brilliant blue R-Prestained molecular weight markers mol wt 26,600–180,000 Da, Goat serum albumin (G9023), lactoferrin from bovine colostrum (L4765), glycine for electrophoresis, and β-mercaptoethanol were obtained from Sigma-Aldrich (Germany). Other chemicals were purchased from POCh (Poland). All chemicals were of analytical grade.

2.2. Equipment

Membrane installation was performed by Poly-Mem (Poland). In addition to a rotary pump, Zuwa

Table 1
Characteristics of the main proteins present in whey [14]

Protein	Concentration (g L^{-1})	Molecular weight (kDa)	Isoelectric pH
β-lactoglobulin (monomer, often present as a dimer)	2.7	18.4	5.2
α-lactoalbumin	1.2	14.1	4.5–4.8
Immunoglobulin G	0.65	150–1,000	5.5–8.3
Serum albumin	0.4	66	4.7–4.9
Lactoferrin	0.1	78	9.0
Lactoperoxidase	0.02	89	9.5
Glycomacropeptides	Varies	<7.0	Various

—Combistar 2000-A, the unit was equipped with a back-flushing system. We applied a back flush of 20 s every 10 min.

The chromatography system was built by ourselves. The unit consisted of a dosing pump (Cole-Parmer, USA), one or two glass columns filled with a resin and a fraction collector (Köhler, Germany).

The analysis equipment used included a spectrophotometer (Hitachi U-1900, Japan), an high pressure liquid chromatography (HPLC) system (Shimadzu, USA) equipment with BIO-SEP SEC-s-2000 and Yara SEC-2000 columns (Phenomenex, USA), and an electrophoresis system (Bio-Rad, USA).

2.3. Analytical procedure

The protein concentration in each fraction was determined by the Lowry method [26] using a standard curve for serum albumin, where C_{protein} (g L^{-1}) = $[(214.1A_{750\ \text{nm}} + 114.6)A_{750\ \text{nm}}]/1{,}000$. The progress of the protein isolation was monitored by electrophoresis in polyacrylamide gels in the presence of SDS [27]. Prestained molecular weight markers, molecular weight 26,600–180,000 Da (Sigma-Aldrich), were used as a standard. After electrophoresis, the gels were analysed using the Bio-Rad Molecular Imager® Gel DocTM XR+ and Image LabTM Software.

The purity of the obtained fractions was analysed in detail by size-exclusion high-pressure liquid chromatography (SE-HPLC) under isocratic conditions using BIO-SEP SEC-s-2000 and Yara SEC-2000 columns serially connected and at an isocratic flow rate of 0.6 mL Min^{-1}. As the eluent, 0.1 M phosphate buffer (pH 6.8) was used. The detection was carried out at a wavelength of 214 nm. All samples were filtered through a 0.45-μm membrane (Chromafil RC-45/15MS, regenerated cellulose). Bovine serum albumin (66.0 kDa), ovalbumin (44.3 kDa), carbonic anhydrase (29.0 kDa), α-lactalbumin (14.2 kDa), cytochrome C (12.4 kDa), aprotinin (6.5 kDa), and vitamin B_{12} (1.3 kDa) were run as standards. The obtained chromatograms were analysed using LabSolutions LC/GC version 5.51 HPLC software (Shimadzu Corporation, Kyoto, Japan).

2.4. Pretreatment procedure

In order to eliminate the natural turbidity of whey (residue of casein clots and fat), the whey was centrifuged (Hettich Zentrifugen Universal 320R, Germany) at 9,000 rpm and 4˚C for 20 min. Then, $CaCl_2$ was added to the whey at 2–5˚C according to the procedure described in the literature [28]. Next, the desired pH was obtained using 6 M NaOH or 2 M HCl. Ionic strength was increased by the addition of solid NaCl. At the end, the whey was heated to 55˚C and held at this temperature for 8 min. After being cooled, the suspension was centrifuged at 9,000 rpm for 20 min.

2.5. Ultrafiltration—concentration of the whey proteins

Two types of membranes were tested: a polymer (PES) with a cut-off coefficient of 80 kDa and a ceramic with a cut-off coefficient of 150 kDa. Before starting the experiments, the membranes were washed for 20 min with 2% NaOH heated to 60˚C and then washed for 30 min with demineralized water. During this procedure, the overpressure was held in order to obtain a permeate.

Further evaluations were conducted at a retentate stream of 0.3 $m^3\ h^{-1}$ using a Zuwa—Combistar 2000 pump at an overpressure of 0.2 MPa. The study was conducted at pH 5.0, 7.3, and 9.0. For the whey at pH 7.3, the effect of ionic strength was also tested. The initial water flow (0.2 MPa, pH 7.3) was 0.121 $m^3\ m^{-2}\ h^{-1}$ for the ceramic membrane and 0.174 $m^3\ m^{-2}\ h^{-1}$ for the polyethersulfone membrane.

During the experiment, the protein concentration was determined by the Lowry method, and chromatographic analyses were performed with a permeate and a retentate stream. The study was conducted for 60–80 min.

2.6. Separation of the lactoferrin with cation-exchange chromatography on CM-Sepharose®

To isolate the lactoferrin, CM-Sepharose® resin was used. Before starting the process, the bed (280 mL) in two columns (450 × 20 mm) was equilibrated with 0.1 mol L^{-1} phosphate buffer pH 7.0. Then, 44 mL of 1.0 mol L^{-1} phosphate buffer pH 7.0 was added to 400 mL of the concentrated whey. The final pH of the whey was 7.0. The whey was passed through the columns. The unadsorbed proteins were washed from the columns using deionized water, and then, the columns were eluted with 0.27 and 0.85 mol L^{-1} NaCl solutions in a stepwise manner at flow rate of 0.5 mL min^{-1}. The protein concentration in the obtained fractions was determined by the Lowry method. The lactoferrin separation efficiency was evaluated using electrophoresis in polyacrylamide gels and SE-HPLC.

2.7. Serum albumin isolation using anion-exchange chromatography on DEAE-Sepharose®

The separation of serum albumin from concentrated goat whey was carried out on DEAE-Sepharose®

(20 mL). The column (115 × 15 mm) was equilibrated with 0.01 mol L^{-1} acetate buffer pH 5.8. The pH of the whey was adjusted to 5.8 with 2.0 mol L^{-1} HCl. After the whey (20 mL) was passed through the column, the column was washed with 0.01 mol L^{-1} acetate buffer, pH 5.8, to remove the proteins that were not adsorbed to the resin. In the next step, the column was eluted with sodium chloride solutions of increasing molarities (from 0.1 to 1.0 mol L^{-1}) at a flow rate of 0.4 mL min^{-1}. The protein concentrations in the obtained fractions were determined by the Lowry method. The separation efficiency was evaluated using electrophoresis in polyacrylamide gels and SE-HPLC.

2.8. Gel filtration

The serum albumin containing fractions obtained from the anion-exchange chromatography were separated on the basis of molecular weight by gel filtration carried out on a column (300 × 15 mm) with Sephadex® G-50 (55 mL). Before this separation, the chromatographic bed was equilibrated with 0.01 mol L^{-1} acetate buffer, pH 6.8 (mobile phase). A 1.0 mL portion of the sample was applied to the top of the column and separated at a flow rate of 0.29 mL min^{-1}. The process was monitored by measuring the protein concentrations in the collected 1 mL fractions and by SE-HPLC of selected fractions.

3. Results and discussion

After the pretreatment procedure, the whey was a clear, yellowish fluid. The protein concentration estimated by the Lowry method varied in the range from 9 to 12 g L^{-1}, and the lactose concentration according to the DNS test was 39–45 g L^{-1}. The dynamic viscosity of this medium at 30℃ was 0.950 mPa s^{-1}. With regard to the molecular weights, the proteins were divided into four subgroups—Fig. 1.

3.1. Ultrafiltration—concentration of the whey proteins

The first step was concentration of the most valuable whey proteins. Good efficiency for the membrane concentration of proteins from dairy effluents has been described previously [29].

The change of permeate flux over time under different process conditions is presented in Fig. 2. Because of the backflushing application, it was possible to maintain the permeate flux at a fairly constant level that was slightly less than the initial ones for the polymeric membrane. This is why only a small amount of protein was deposited on the membrane, which in a particular experiment was in the range from 3.3 to 4.4 g m^{-2}. This value for the ceramic membrane was a few times higher (from 11.5 to 26.9 g m^{-2}), and in this case, the decrease in the permeate stream was also much higher.

The protein concentrations in the permeate and retentate streams were determined by the Lowry method [26]. Determined using Eq. (1) [30,31], the values of the retention coefficient under the specified process conditions are shown in Fig. 3. The results consider the values determined after 60 min of the process run. Their values did not change significantly over time.

$$R_i = \frac{C_{i,permeate}}{C_{i,retenate}} \tag{1}$$

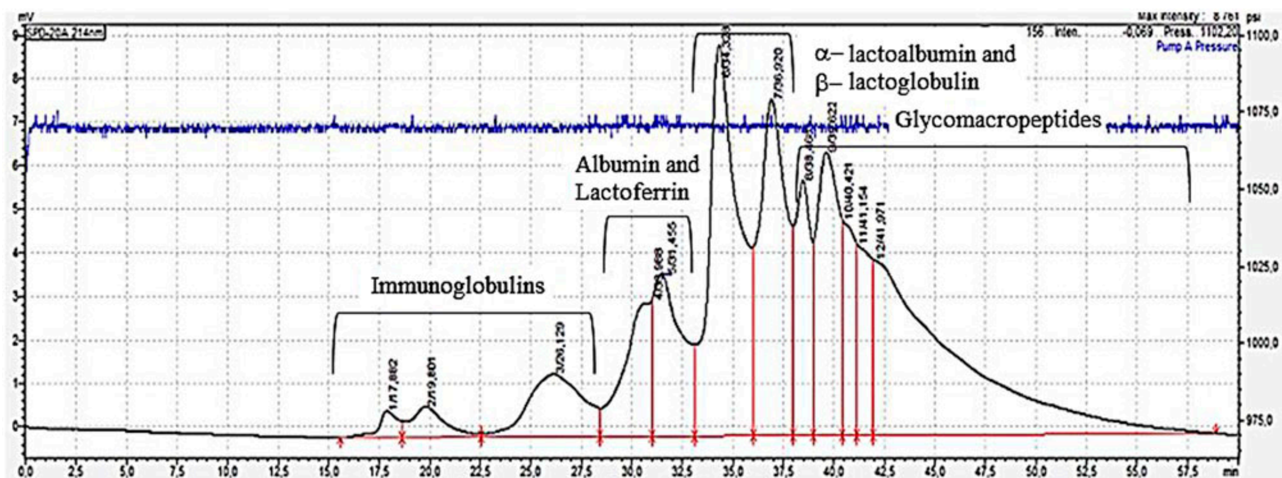

Fig. 1. Proteins present in whey.

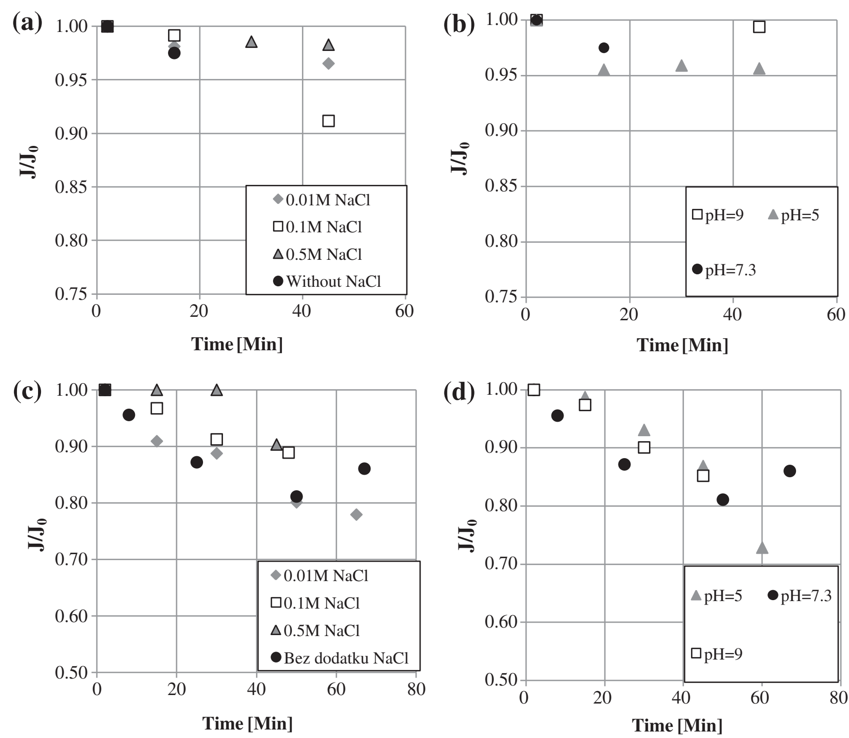

Fig. 2. Influence of ionic strength and pH on the decrease in the permeate stream during the separation on the polymeric (a and b) and the ceramic (c and d) membranes.

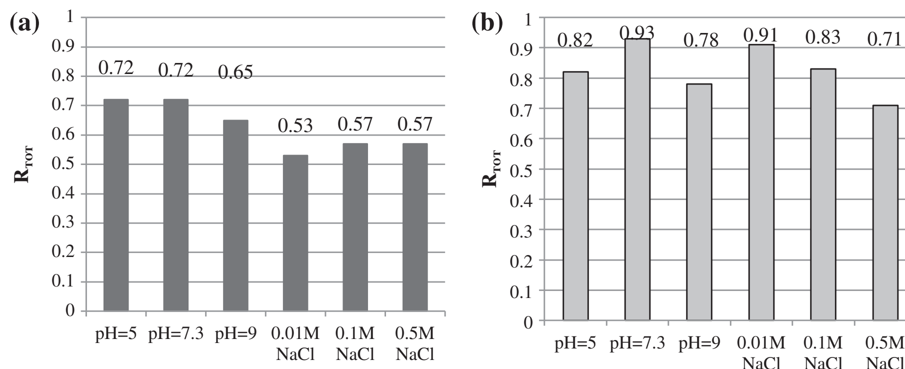

Fig. 3. Values of the retention coefficient with respect to the total amount of whey proteins on the polymeric (a) and ceramic (b) membranes.

An increase in the ionic strength for both tested membranes resulted in a decrease in protein retention. However, for the ceramic membrane, the change was smaller and increased with an increase in ionic strength. In the case of the polyethersulfone membrane, a slight increase in ionic strength resulted in a significant decrease in the retention coefficient. Raising the pH to 9.0 for both membranes resulted in a lower retention rate. The analysis of the chromatograms allowed us to determine the retention of individual groups (divided based on the molecular weight) of whey proteins—Fig. 4.

The decrease in the retention coefficient expressed based on the total protein mass resulted from a decrease in the retention of the smallest whey proteins—glycomacropeptides, α-lactoalbumin, and β-lactoglobulin. From the point of view of further use of the most valuable whey proteins (serum albumin and

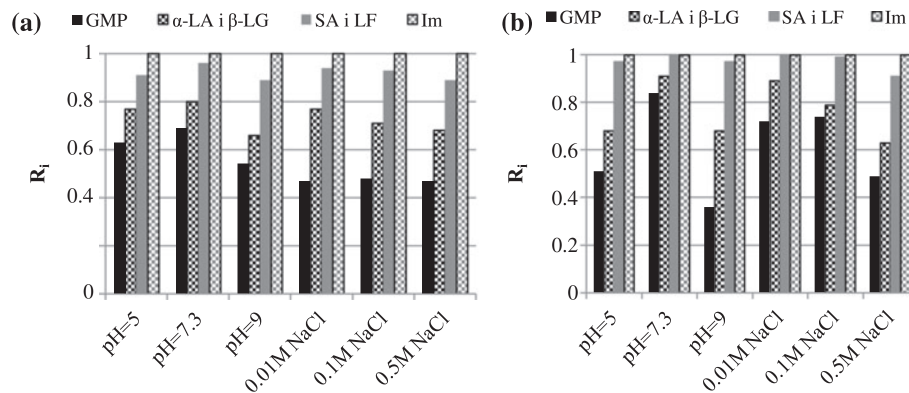

Fig. 4. Influence of pH and ionic strength on the retention coefficient of the individual groups of whey proteins on the polymeric (a) and ceramic (b) membranes.

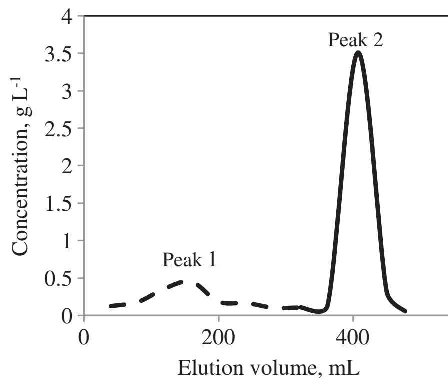

Fig. 5. Elution profile of lactoferrin separation by cation-exchange chromatography using two columns (450 × 20 mm) with 280 ml of CM-Sepharose. Input: 444 mL concentrated whey (pH 7.0); washing phase: deionized water; elution phase: 0.27 M NaCl solution (dashed line) and 0.85 M NaCl solution (solid line) in a stepwise manner.

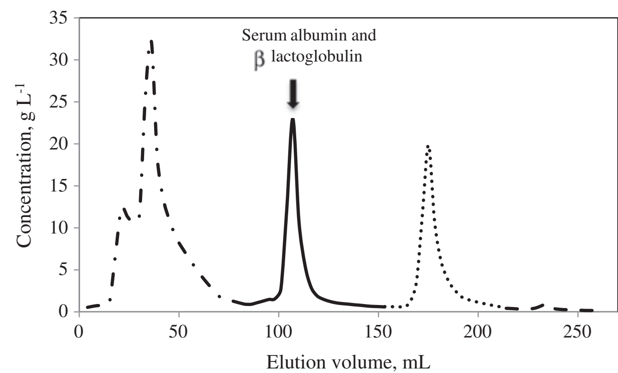

Fig. 6. Elution profile of albumin serum separation on a column (115 × 15 mm) with DEAE-Sepharose (20 mL). Input: 20 mL concentrated whey (pH 5.8); washing phase: 0.01 M acetic buffer pH 5.8; elution phase: 0.1 M NaCl solution (dotted-dashed line), 0.2 M NaCl solution (solid line), 0.3 M NaCl solution (dotted line) and 0.4 M NaCl solution (dashed line).

lactoferrin), the process using the ceramic membrane and separation at pH 9.0 was the best (full retention of these proteins and low glycomacropeptides, α-lactoalbumin, and β-lactoglobulin). However, working with the polymer membrane provides a low degree of adsorption of proteins and a steady stream of permeate. That is why the separation on this membrane at a slightly increased ionic strength (0.01 M NaCl) was chosen. By including diafiltration with ultrafiltration, the glycomacropeptides could be diluted completely. This low ionic strength did not influence the following chromatographic separation steps.

3.2. Isolation of lactoferrin using a cation-exchange resin

Lactoferrin has an isoelectric point of 7.9 (Table 1) and thus can be isolated using a cation-exchange resin. The separation principle consists of the selective

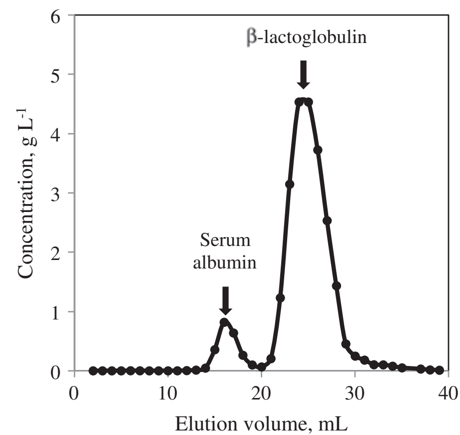

Fig. 7. Elution profile of gel filtration carried out on Sephadex® G-50 (55 mL) for the fraction (1.0 mL) obtained during anion-exchange chromatography on DEAE-Sepharose and washed with 0.2 M NaCl solution.

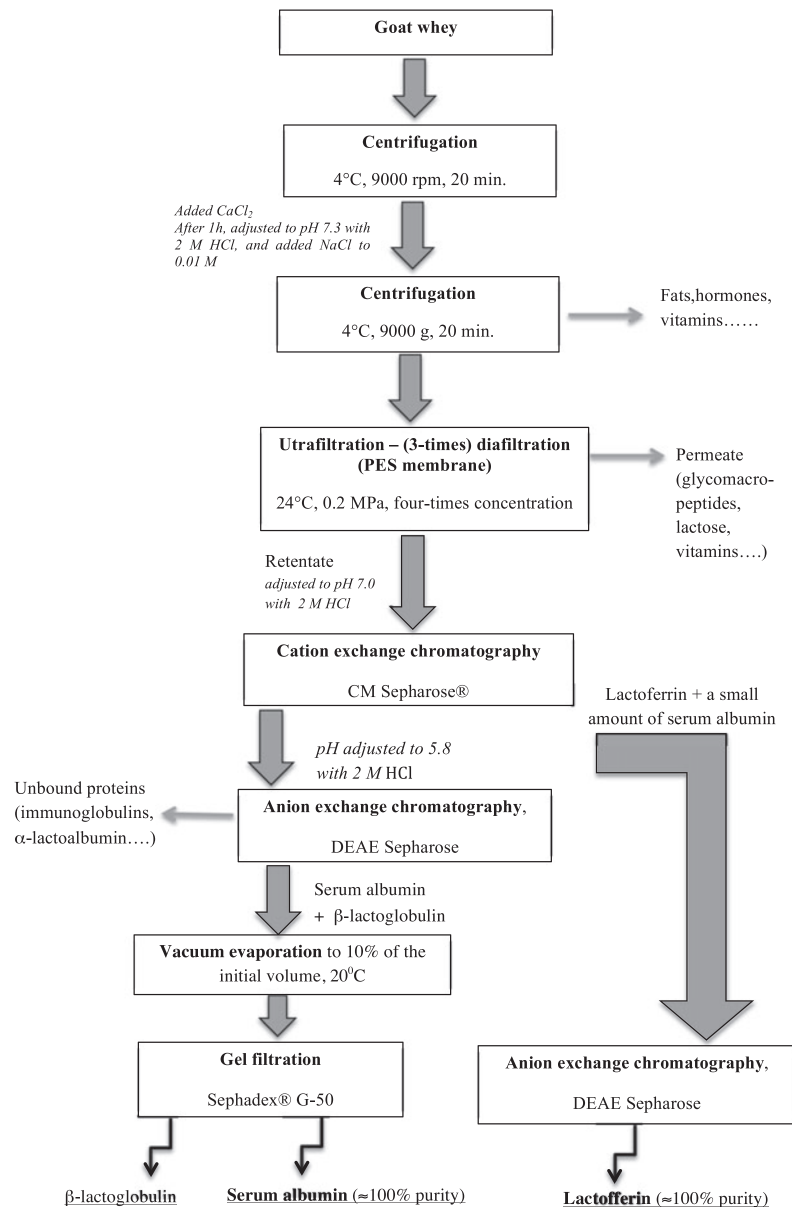

Fig. 8. Protocol for isolation of lactoferrin and serum albumin from goat whey.

adsorption of proteins with a pI greater than 7.0 to the CM-Sepharose bed, followed by selective elution using sodium chloride solutions of increasing molarities. Other proteins present in the whey have pIs significantly below this value and as expected did not bind to the matrix. The elution profile of the cation-exchange chromatography is shown in Fig. 5. The weakly adsorbed proteins that eluted with a 0.27 mol L^{-1} NaCl solution are represented by Peak 1 on the chromatogram. Using SDS-PAGE electrophoresis, we identified the presence in this fraction of β-lactoglobulin, immunoglobulin heavy chain and possibly lactoperoxidase (because the lactoferrin molecular weight is close to that of lactoperoxidase, distinguishing these two proteins is difficult). The second peak (Peak 2) includes proteins that were more strongly adsorbed onto the resin and were eluted with the 0.85 mol L^{-1} NaCl solution. Lactoferrin was the predominant protein observed in this peak. Surprisingly, a small amount of serum albumin was also detected in this fraction. The mass balance indicates that the overall recovery of protein was 99%.

3.3. Isolation of serum albumin on an anion-exchange resin

Proteins with an isoelectric point below the local pH should bind to an anion-exchange resin. This property was used to separate serum albumin using DEAE-Sepharose at pH 5.8. The column was eluted with sodium chloride at concentrations in the range of 0.1–1.0 mol L^{-1}. The fractions eluted with NaCl solutions with concentrations greater than 0.4 mol L^{-1} were characterized by negligible protein concentrations. Fig. 6 therefore shows the elution profiles for fractions eluted with sodium chloride at concentrations from 0.1 to 0.4 mol L^{-1}. The SE-HPLC chromatogram of the fraction eluted at 0.2 mol L^{-1} showed two peaks, representing serum albumin and β-lactoglobulin. The molecular weights of these proteins indicated that they could be separated by gel filtration.

3.4. Final purification

Using the fraction that included serum albumin (Fig. 6), gel filtration on Sephadex® G-50 was performed. The obtained elution profile (Fig. 7) showed two distinct peaks, which were identified by SE-HPLC as serum albumin and β-lactoglobulin. The purity of the serum albumin was 98.98%. The two-step procedure resulted in recovery of 91.7% of the serum albumin.

Because serum albumin has a high affinity for DEAE-Sepharose, this resin was used for the final purification of the fraction obtained from the CM-Sepharose (Fig. 5). Lactoferrin (the dominant protein) did not bind to the DEAE column at pH 5.8, while serum albumin (the major impurity) strongly bound to this matrix. The SE-HPLC analysis showed that the LF fraction had a purity of greater than 99%. The final recovery of the LF was 93.2%.

4. Protocol for the lactoferrin and serum albumin isolation

Fig. 8 shows a diagram of the multistage process for the isolation of the goat whey proteins of medical importance with the required high (≅100%) degree of purification of the proteins. On the basis of the experiments performed at the laboratory scale, we propose a six-stage separation process based on centrifugation, ultrafiltration accompanied by diafiltration, cation and anion-exchange chromatography, vacuum evaporation and gel filtration. In order to minimize the required volume of the Sephadex® G-50, vacuum evaporation to reduce the volume by 90% was implemented before the gel filtration. Overall, the volumes of the resins required in the proposed technology are very large. However, it should be noted that these resins can be used multiple times by regeneration with buffers.

According to the mass balance as indicated by the laboratory-scale preparations, the recoveries of lactoferrin and serum albumin are assumed to be 93.2 and 91.7%, respectively. Assuming the LF concentration in the starting medium to be approximately 0.1 g L^{-1}, it is possible to obtain approximately 93 g of lactoferrin from 1 m^3 of crude whey. Similarly, at an initial concentration of 0.4 g L^{-1}, 367 g of serum albumin could be obtained in one cycle.

5. Conclusions

By performing the integrated process of whey treatment, we isolated the most important proteins: lactoferrin and serum albumin, and additionally, we separated β-lactoglobulin. The cost of obtaining a given quantity of a protein at an analytical grade is high, primarily due to the low concentrations of the substance in the initial medium. Therefore, the advanced purification of the proteins for direct use in specific medical treatments or for therapies using peptides derived from their controlled hydrolysis is addressed by the proposed process.

The side streams generated by this process could be used for animal feed. This product could contain the unused upper fraction of the centrifugation (including fat, hormones, vitamins, glycomacropeptides, lactose and water-soluble vitamins) derived from the permeate and the proteins not bound to the DEAE-Sepharose. In this way, the proposed technology would meet the requirements of the so-called clean (waste-free) technology.

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