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Bovine whey fractionation based on cation-exchange chromatography[1]

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Abstract

Bovine whey proteins have potential applications in veterinary medicine, food industry and as supplements for cell culture media. A fractionation scheme for the economically interesting proteins, such as IgG, lactoferrin and lactoperoxidase, based on cation exchangers was the goal of our investigations. A chromatographic process was developed where α-lactalbumin passes through the column and separation of the desired proteins is achieved. Four different cation-exchange media (S-HyperD-F, S-Sepharose FF, Fractogel EMD SO_3^- 650 (S) and Macro-Prep High S Support) were compared in regard to their dynamic binding capacity for IgG and their different elution behaviours when sequential step gradients with NaCl buffers were applied. Peak fractions were analyzed by size-exclusion chromatography and sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Lactoperoxidase activity was monitored by the oxidation of *o*-phenylenediamine. In order to explain the different resolution behaviours, isocratic runs with pure standards of whey proteins were performed. The k' values were calculated and plotted against salt concentration. Fractogel EMD had the highest binding capacity for IgG, 3.7 mg/ml gel at a linear flow-rate of 100 cm/h, but the resolution was low compared to that with the other three media. S-Hyper D and S-Sepharose FF showed lower capacities, 3.3 and 3.2 mg/ml gel, respectively, but exhibited better protein resolution. These effects could be partially explained by the k' versus salt concentration plots. The binding capacity of Macro-Prep S was considerably lower compared to that of the other resins investigated because its selectivity for whey proteins was completely different. S-Sepharose FF and S-Hyper D combine relatively high dynamic capacity for IgG and good resolution. Compared to studies with standard proteins, such as 100 mg/ml bovine serum albumin for S-Hyper D, their binding capacities were very low. Even after removal of low-molecular-mass compounds, the capacity could not be improved significantly. The running conditions (low pH) were responsible for the low protein binding capacity, since low-molecular-mass compounds in the feed do not compete with the adsorption of whey protein. The dynamic capacity did not decrease to a large extent within the range of flow-rates (100–600 cm/h) investigated. The dynamic capacity of HyperD and Fractogel was at least five times higher when pure bovine IgG was used for determination. In conclusion, S-Sepharose FF, S-Hyper D-F and Fractogel EMD SO_3^- 650 (S) are considered as successful candidates for the large-scale purification of bovine whey proteins.

Keywords: Whey; Binding capacity; Preparative chromatography; Proteins; Lactoferrin; Immunoglobulins

1. Introduction

Bovine whey contains numerous proteins that are of interest for veterinary medicine and human and

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animal nutrition [1,2]. Whey is available in large amounts, since it is a by-product of cheese manufacturing. IgA, IgG, lactoferrin and lactoperoxidase, present in bovine whey, have high pharmaceutical value. Oral administration of bovine IgG is known to be an effective treatment of various infections for new-born infants [3–5]. Lactoferrin and lactoperoxidase are known to act as antimicrobial factors [6,7]. Therefore, effective whey protein processing is an important engineering task.

Efficient whey processing is also required because of recent developments in the expression of recombinant proteins under the control of the β-lactoglobulin promoter [8]. These recombinant proteins are secreted into milk and have to be isolated from this feedstock. Casein precipitation and initial recovery of the recombinant protein from whey is an important step in this process [9]. Further purification cycles must be performed according to the requirements of each individual protein overexpressed into milk. The initial purification sequence of recombinant proteins secreted from milk glands exhibits similarities to the purification sequence applied for certain whey proteins.

Several preparative chromatographic methods have been reported for whey protein fractionation. Anion-exchange chromatography on QMA-Spherosil has been used to concentrate whey proteins and separate them from lactose, by elution of the bound proteins using hydrochloric acid [10]. β-Lactoglobulin and α-lactalbumin have been separated on diethylaminoethyl (DEAE)–cellulose and DEAE–toyopearl [11–13]. The intention of these investigations was the biochemical characterisation of protein variants. Lactoferrin and lactoperoxidase were separated on carboxymethyl cellulose after ammonium sulphate precipitation of whey [9]. IgG, lactoferrin and lactoperoxidase could also be separated by several gel filtration techniques [14]. Due to the ability of lactoferrin to bind DNA, it could also be purified by DNA affinity chromatography [15]. Recent publications showed that lactoferrin acts as a transcription factor, which explains its DNA-binding affinity [16]. IgG was purified from bovine whey using thiophilic gels [17], immobilized metallized dye chromatography [18] or chelating chromatography [19], with reasonable yields but low productivity. Therefore, the large-scale application of these methods is limited.

For concentration of whey proteins, acidic whey (pH<4.5) was adsorbed on the cation-exchange resin, Spherosil S, and proteins were eluted with buffers with higher pH values [20]. Other cation-exchange chromatography techniques were used for analytical whey characterisation [21,22]

Cation-exchange chromatography has not been reported as the initial step for fractionation of whey proteins, after casein removal. Several cation-exchange resins have been improved regarding physico-chemical and mechanical properties and designed for industrial protein purification, mainly in the area of biopharmaceuticals. Our objective was to use these sorbents for an application that they had not been designed for. Four different cation-exchangers, representing members of the various types of chromatography matrices, have been selected for investigation. Experiments have been carried out to characterize the properties of each resin, in particular, dynamic binding capacity for IgG and selectivity. Conventional acid precipitation of delipidated bovine milk was used for production of the feed stock. Conditions where lactalbumin passes through the column and proteins with higher isoelectric points (p*I*) than lactalbumin are retained on the column have been considered as starting points for the development of a purification strategy. By following these constraints, the methods should be suited for large scale operation.

2. Experimental

2.1. Chromatography materials

Macro-Prep High S Support, S-Sepharose Fast Flow and S-HyperD-F were gifts from Bio-Rad (Richmond, CA, USA), Pharmacia Biotech (Uppsala, Sweden) and Biosepra (Marlborough, MA, USA). Fractogel EMD SO_3^- 650 (S) was purchased from Merck (Darmstadt, Germany).

2.2. Pre-treatment of milk

Milk was centrifuged in a Beckmann J2-21 centrifuge equipped with a JA-20 rotor (Beckman instruments, Palo Alto, CA, USA) at 4420 *g* at room temperature for 30 min for delipidation. The pH of the skimmed milk was adjusted to 4.7 by the slow

addition of 5 *M* HCl. After casein precipitation, the solution was stirred for a further 30 min to complete precipitation. Casein was removed by centrifugation at 17 700 *g* and 4°C for 30 min. The obtained whey was diluted with distilled water until a conductivity of 2.7 mS/cm was obtained. The pH was readjusted to 4.7, since the pH shifted during dilution. Prior to chromatography, the whey was filtered through a 0.45-μm Millipak-60 filter (Millipore, Bedford, MA, USA).

2.3. Preparative cation-exchange chromatography

A fast protein liquid chromatography (FPLC) system (Pharmacia Biotech), consisting of two P-500 pumps, two MV 8 motor valves, an MV 7 injection valve, a dual path monitor UV-2, a 5-MPa mixer and a 2195 pH/ion monitor, controlled by an LCC 500 controller, was used. The cation-exchange resins were packed into HR 10 and HR 16 columns (Pharmacia). Column dimensions were 30/10 and 35/16 mm (height×I.D.), respectively. The buffers were prepared from citric acid and the pH was adjusted with 1.0 *M* NaOH. The cation-exchangers were regenerated with 1 *M* NaCl in 20 m*M* citric acid and equilibrated with 20 m*M* citric acid, pH 4.7, 2.7 mS/cm. Chemicals for buffer preparation were purchased from Merck. After loading and washing with equilibration buffer, the bound proteins were eluted with sequential gradients with increasing NaCl concentrations.

2.4. Analytical size-exclusion chromatography (SEC)

SEC was performed on a 2150 HPLC pump (LKB, Bromma, Sweden). A Superdex 200 HR10/30 column (Pharmacia) was used. UV detection at 214 nm was carried out on a 2151 variable-wavelength monitor (LKB). A 50-μl volume of each sample was applied using an autosampler 1050 from Hewlett–Packard (Waldbronn, Germany). Proteins were eluted with 0.1 *M* sodium phosphate buffer, pH 7.0, containing 0.15 *M* NaCl at a flow-rate of 1 ml/min. UV signals were processed on a Turbochrom chromatography workstation 3.1 (PE Nelson Systems, Cupertino, CA, USA). A calibration curve for bovine IgG (Sigma, St. Louis, MO, USA) between 10 and 150 μg/ml was determined.

2.5. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

Fractions were analyzed under non-reducing conditions with 4–20% acrylamide gradient gels using an Xcell mini-cell, both purchased from Novel Experimental Technology (San Diego, CA, USA). Samples were diluted, SDS was added to a final concentration of 2%, and samples were boiled for 10 min prior to electrophoresis. The final protein concentration used was approximately 500 μg/ml. A 15-μl volume of each sample was loaded. Protein staining was performed with Coomassie Brilliant Blue R 250, 0.5% (w/v) in 10% acetic acid and 25% methanol. Destaining was carried out in 10% acetic acid and 25% methanol.

2.6. Bovine IgG enzyme-linked immunosorbent assay (ELISA)

Bovine IgG content was determined by a double antibody sandwich ELISA. Rabbit anti-bovine IgG (Sigma) was coated on microtitre plates. After coating and washing, the samples were applied to the wells. Eight 2^n dilutions were made from samples and standard. Bovine IgG standard (Sigma) was used in a range from 400 to 3.125 ng IgG/ml. As the second antibody, rabbit anti-bovine IgG conjugated with horseradish peroxidase (Sigma) was used. After staining with *o*-phenylenediamine (Sigma), adsorption was measured at 492 nm. The results were evaluated using a fourth degree polynomial.

2.7. Dynamic binding capacity for IgG

The dynamic binding capacity for IgG from whey at 10% breakthrough was calculated from the volume of sample loaded minus the void volume of the column and the volume of the inlet/outlet tubing. IgG content of unbound fractions was analyzed by ELISA and SEC. Different linear flow velocities were investigated. Purified bovine IgG (reagent grade) purchased from Sigma was also used for determination of dynamic capacity.

2.8. Determination of k′ values

Isocratic runs with the proteins of interest present in bovine whey (α-lactalbumin, β-lactoglobulin,

bovine IgG, lactoperoxidase and lactoferrin; Sigma) were carried out at different salt concentrations. The four chromatography resins were packed into 10/0.5 cm (height×I.D.) columns and operated at 150 cm/h. Samples (100 μl) with a protein concentration of 2 mg/ml were applied. The signal of the UV monitor was recorded by an electronic data acquisition system (Nelson analytical interface 760 series, model 2600 chromatography software, rev.5.0; PE Nelson Systems). Chromatographic data were analyzed by statistical moments using the software Peak Fit, version 4.0 (Jandel Scientific, Erkrath, Germany). The first moment was calculated and used as a measure of the retention time (t_R). The k' value has been defined as

$$k' = \frac{t_R - t_0}{t_0}$$

where t_R is the elution time and t_0 is the dead time, which has been defined as the elution time of the respective protein under non-binding conditions. The log k' value was plotted against log (1/[NaCl]) according to Kopaciewicz et al. [25].

2.9. Determination of lactoperoxidase activity

Lactoperoxidase activity was determined by a modified method described by Putter and Becker [24]. *O*-Phenylenediamine (OPD) was used instead of 2,2′-azinobis[3-ethyl-benzothiazoline-6-sulphonic] diammonium salt (ABTS) as substrate for the enzyme.

2.10. Desalting by SEC

A 450-ml volume of Sephadex G-25, coarse medium (Pharmacia), packed into an XK-50 column (Pharmacia), was equilibrated with 20 m*M* citric acid buffer, pH 4.7. A 120-ml volume of undiluted whey was loaded and desalted.

2.11. Desalting by ultra/diafiltration

A 150-ml volume of undiluted whey was concentrated to 30 ml in a 67-mm stir cell equipped with a M_r 30 000 cut-off ultrafiltration membrane (YM 30 Diaflo membrane) from Amicon (Danvers, MA, USA), and diafiltered against 300 ml of citric acid buffer, pH 4.7.

3. Results

3.1. Initial screening of appropriate binding conditions

The objective of the study was the development of a fractionation scheme for bovine whey that allowed the binding of IgG and proteins with p*I* values higher than that of α-lactalbumin, to a cation-exchange resin. Lactalbumin should pass through the column. According to the known isoelectric points of the major proteins present in whey (Table 1), a pH value above 4.7 was chosen for detailed investigation. The whey prepared as described in Section 2 was adjusted to different pH values (pH 4.7, 4.9, 5.1 and 5.4). These solutions were loaded onto the cation exchangers described in Section 2. Fractions were collected from the flow-through and the IgG concentration was determined by analytical SEC and ELISA. In all cases when the pH was higher than 4.7, IgG was detected in the early fractions, indicating that, under these conditions, IgG did not bind significantly to the resin (data not shown). These binding characteristics were observed with all of the cation exchangers that were selected for this study. When whey was diluted with distilled water to adjust the conductivity to 2.7 mS/cm, IgG bound to the cation-exchange column equilibrated with a 20 m*M* citric acid buffer, pH 4.7. Again, fractions from the flow-through were collected and subjected to SEC

Table 1
Protein composition of bovine whey according to McKenzey [23]

Protein	Average concentration in whey (g/l)	Molecular mass ($\times 10^{-3}$)	Isoelectric point, p*I*
β-Lactoglobulin	3–4	18.4	5.2
α-Lactalbumin	1.5	14.2	4.7–5.1
Serum albumin	0.3–0.6	69	4.9
IgG, IgA, IgM	0.6–0.9	150–900	5.8–7.3
Lactoperoxidase	0.06	78	9.6
Lactoferrin	0.05	78	8.0
Proteose-peptone	0.5	4–20	

Only major whey proteins have been listed.

analysis and ELISA (Figs. 1–3). SEC data are shown from fractions at the beginning of loading and fractions when IgG started to break through. The respective fractions have been denoted as S-Sepharose 1, 2; S-HyperD 1, 2; Macroprep S 1, 2 and Fractogel 1, 2.

The flow-through contained mainly α-lactalbumin and small amounts of β-lactoglobulin. The protein composition of the flow-through varied with different cation-exchangers, as seen by the different peak patterns found on SEC analysis (Fig. 3).

3.2. Elution conditions

It was intended to employ step gradients for elution, since the method should be easily transferable to large scale. Sequential gradients with 0.1, 0.2, 0.3, 0.4 and 1.0 *M* NaCl were used to study the elution behaviour of the proteins bound under the optimized conditions. The elution profiles are shown in Fig. 1. The eluted fractions were analyzed by SDS–PAGE and the proteins were identified by their electrophoretic mobilities. The chromatographic media showed different selectivities and, therefore, also different resolution for the whey proteins bound during loading (Figs. 2–4, Table 2).

The majority of IgG could be eluted with 0.1 *M* NaCl from S-HyperD. The eluate also showed high lactoperoxidase activity. At 0.3 *M* NaCl, lactoferrin and small amounts of IgG were eluted. With 1 *M* NaCl, the remaining traces of lactoferrin and lactoglobulin were removed (Fig. 1A, Fig. 4A, lanes 6–9).

On Macro-Prep S, the 0.1 *M* NaCl fraction

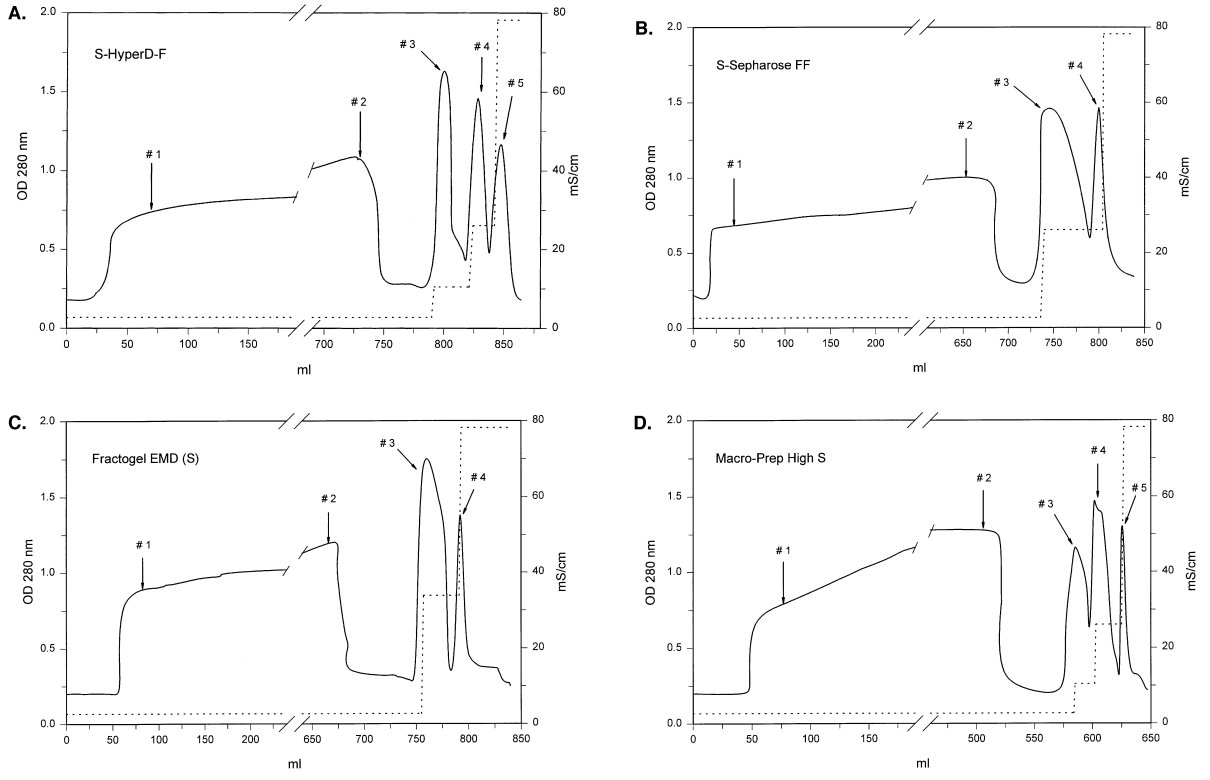

Fig. 1. Purification of bovine whey proteins by cation-exchange chromatography. Clarified whey was applied to four different cation-exchange columns (3.5×1.6 cm), equilibrated with 20 m*M* citric acid, pH 4.7. The flow-rate was 3.3 ml/min (200 cm/h). Elution of bound material was carried out with sequential NaCl gradients. Unbound material and eluted fractions were characterized by analytical size-exclusion chromatography and SDS–PAGE (Figs. 3 and 4). (A) S-HyperD-F, (B) S-Sepharose FF, (C) Fractogel EMD SO_3^- S, (D) Macro-Prep High-S Support (·····, theoretical salt gradient; ———, UV absorbance at 280 nm).

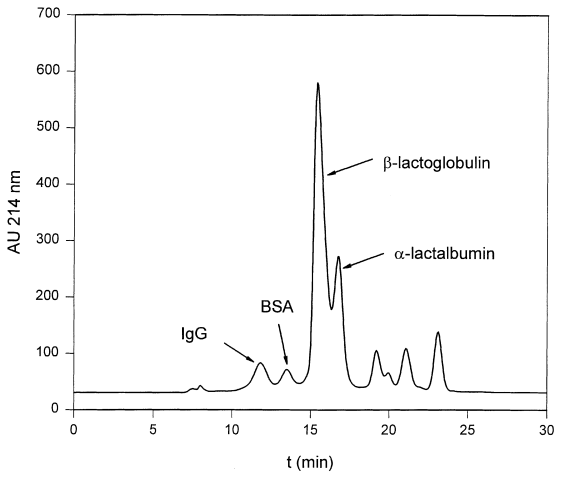

Fig. 2. Analytical size-exclusion chromatography on Superdex 200. A 50-μl volume of diluted bovine whey (starting material) was applied to the column and eluted with 0.1 *M* sodium phosphate buffer, pH 7.0, containing 0.15 *M* NaCl.

contained mainly β-lactoglobulin and some IgG. With 0.3 *M* NaCl, IgG and lactoferrin were eluted and high lactoperoxidase activity was detected. High enzyme activity was defined by an absorbance of 1.5 at a dilution of 1:10 (v/v) after incubation for 15 min. Actual enzyme units have not been determined. At 1.0 *M* NaCl, the remaining IgG was recovered (Fig. 1B, Fig. 4B, lanes 2–4).

S-Sepharose FF and Fractogel EMD (S) showed considerable differences in elution behaviour compared to the aforementioned chromatographic media. Elution with NaCl concentrations below 0.3 *M* produced broad peaks with large tailing, resulting in unacceptably large elution volumes. IgG could be eluted from S-Sepharose with 0.3 *M* NaCl and from Fractogel with 0.4 *M* NaCl. Both fractions contained much more lactoglobulin compared to the eluates from S-HyperD and Macro-Prep. Lactoperoxidase activity could be detected in these fractions. Lactoferrin was eluted with 1.0 *M* NaCl, but the eluate from S-Sepharose exhibited higher purity (Fig. 1C,D, Fig. 4B, lanes 5–10). The protein composition of the eluted fractions is summarized in Table 2.

3.3. Dynamic binding capacity for IgG

Dynamic binding capacity was determined by 10% breakthrough of IgG. In Fig. 5, the dynamic binding capacities for IgG at different linear flow-rates are summarized. Compared to pure standard proteins, the capacity for IgG seems to be low. Low-molecular-mass impurities are thought to have been responsible for this low capacity. Therefore, two attempts were made to increase the capacity: low-molecular-mass substances were removed either by gel filtration on Sephadex G-25 or by ultra/diafiltration using a membrane with a M_r cut-off of 30 000. Subsequently, the pre-treated whey was applied to the cation-

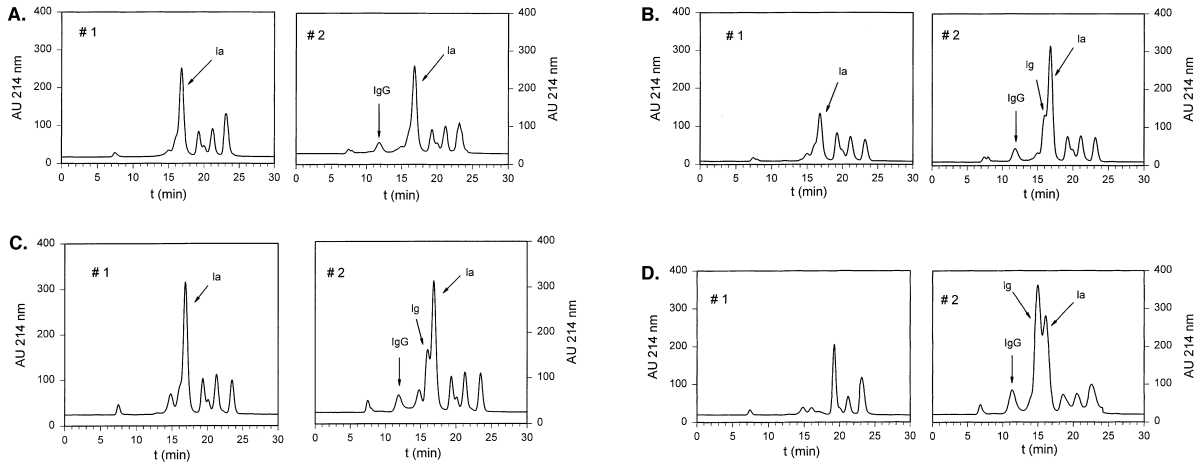

Fig. 3. Analytical size-exclusion chromatography on Superdex 200 of unbound material (flow-through) when bovine whey was loaded onto cation-exchange sorbents. Fractions of the flow-through, as indicated in Fig. 1, were analyzed. (A) S-HyperD-F 1 and 2, (B) S-Sepharose FF 1 and 2, (C) Fractogel EMD SO_3^- S 1 and 2, (D) Macro-Prep High-S Support 1 and 2.

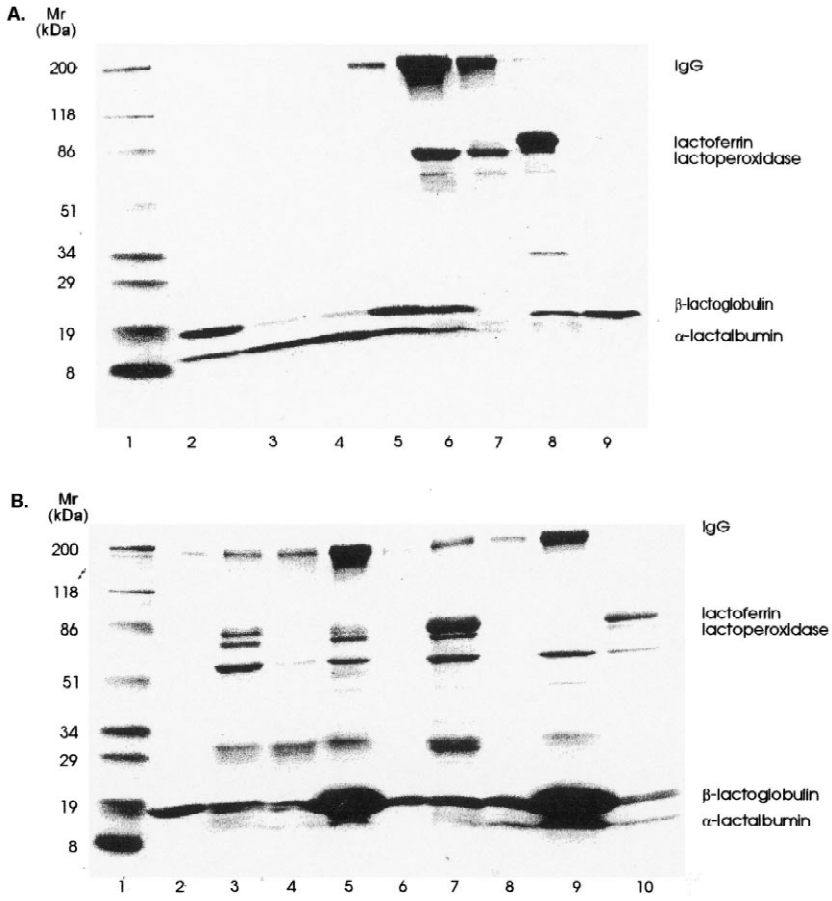

Fig. 4. SDS–PAGE profile of diluted whey and fractions obtained by cation-exchange chromatography. (A) lane 1, molecular mass markers; lane 2, diluted whey (starting material); lanes 3 and 4, S-HyperD 1 (early flow-through); lane 5, S-HyperD 2 (flow-through at the end of the loading step); lane 6, S-HyperD 3 (0.1 *M* NaCl eluate); lane 7, S-HyperD 3 (0.1 *M* NaCl eluate); lane 8, S-Hyper D 4 (0.3 *M* NaCl eluate) and lane 9, S-HyperD 5 (1.0 *M* NaCl eluate). (B) Lane 1, molecular mass markers; lane 2, Macro-Prep S 3 (0.1 *M* NaCl eluate); lane 3, Macro-Prep S 4 (0.3 *M* NaCl eluate); lane 4, Macro-Prep S 5 (1.0 *M* NaCl eluate); lane 5, Fractogel EMD S 3 (0.4 *M* NaCl eluate); lane 6, Fractogel EMD S 3 (0.4 *M* NaCl eluate); lane 7, Fractogel EMD S 4 (1.0 *M* NaCl eluate); lane 8, S-Sepharose FF 3 (0.3 *M* NaCl eluate); lane 9, S-Sepharose FF 3 (0.3 *M* NaCl eluate) and lane 10, S-Sepharose FF 4 (1.0 *M* NaCl eluate). Samples were taken as indicated by the arrows in Fig. 1A–D.

exchangers and the dynamic binding capacity was determined. Both pre-treatment methods did not improve the binding capacity. A second explanation for the low binding capacity was the overall protein concentration. A starting solution with a similar protein composition but an extremely high IgG content was used to examine the dynamic binding capacity further.

Whey prepared from colostral milk in the same manner as described for regular milk was an optimal starting solution for this purpose. Analytical SEC showed IgG as the predominant protein component (Fig. 6). This solution contained 7 mg IgG/ml in comparison to 0.4 mg/ml present in diluted regular whey. Hence, S-Sepharose and S-HyperD exhibited a capacity in the range of 4.5 mg IgG/ml gel. From these results, we concluded that the sorption capacity for IgG under these conditions is in the observed range (Fig. 7). Furthermore, purified bovine IgG has been used for determination of the dynamic capacity under buffer conditions that were optimal for the required resolution of the different whey proteins

Table 2
Qualitative protein composition of eluted fractions

Cation-exchanger	Salt concentration in elution step			
	0.1 *M* NaCl	0.3 *M* NaCl	0.4 *M* NaCl	1 *M* NaCl
S-HyperD-F	IgG, lactoperoxidase, low lactoglobulin content	Lactoferrin, lactoperoxidase	Not done	Traces of all whey proteins
S-Sepharose FF	Proteins do not elute	IgG, lactoperoxidase, significant lactoglobulin and lactalbumin	Not done	Lactoferrin, lactoperoxidase
Fractogel EMD (S)	Proteins do not elute	Proteins do not elute	IgG, lactoperoxidase sign. lactoglobulin	Lactoferrin, IgG
Macro-Prep High S	Low lactoglobulin and low IgG content	IgG, lactoferrin, lactoperoxidase, low lactoglobulin content	Not done	IgG, traces of other proteins

(pH, 4.7; conductivity, 2.7 mS/cm). The dynamic binding capacity of Macro-Prep S and S-Sepharose was in the same order of magnitude, regardless of the feedstock used. Fractogel EMD (S) and S-HyperD showed different behaviours. The capacity determined with purified IgG exceeded the value obtained with whey from milk or colostrum (Table 3).

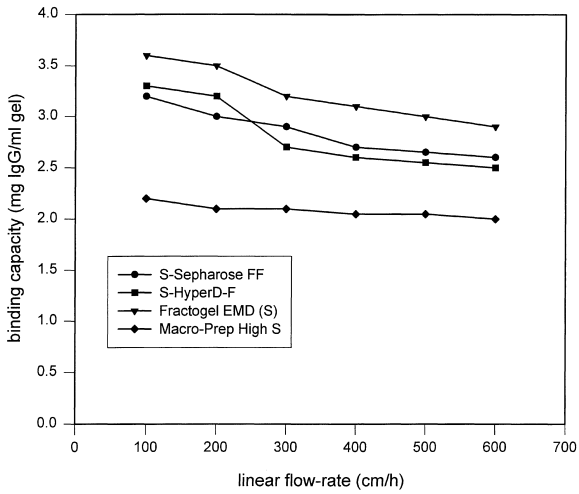

Fig. 5. Dynamic binding capacity for IgG from bovine whey at 10% break-through. The IgG concentration was determined by analytical size-exclusion chromatography and ELISA.

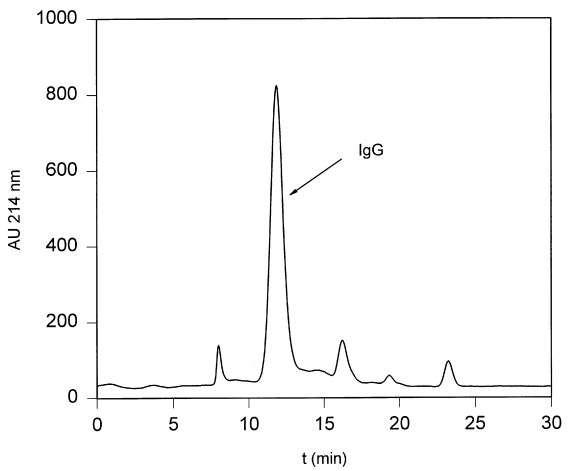

Fig. 6. Analytical size-exclusion chromatography on Superdex 200 of whey prepared from colostral milk.

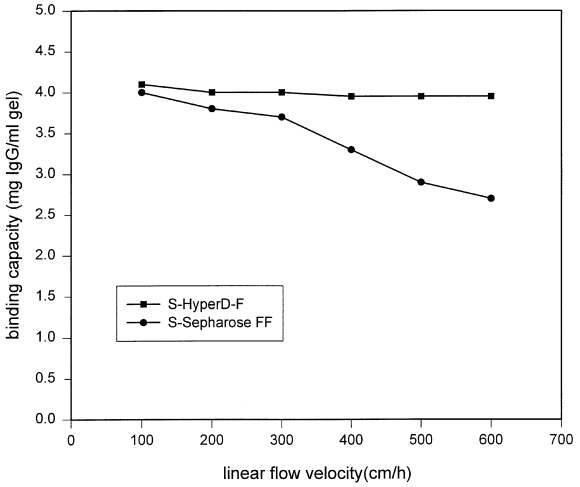

Fig. 7. Dynamic binding capacity for IgG from whey prepared from colostral milk. IgG concentration was determined by analytical size-exclusion chromatography and ELISA.

3.4. Isocratic elution behaviour of single whey proteins

In order to explain the different selectivities of the four resins, the k' values of α-lactalbumin, β-lactoglobulin, bovine IgG, lactoperoxidase and lactoferrin were determined under different salt concentrations, as described in Section 2. Data are shown in Fig. 8. Incomplete isocratic elution was observed with S-HyperD. A certain fraction of the injected protein always remains bound to the resin. Therefore, the data set describing S-HyperD has to be interpreted carefully. The shape of the plots is completely different compared to that found for the other sorbents. We made a linear extrapolation of k' values towards higher salt concentration. In the case where an angled curve was observed (lactoperoxidase chromatographed on S-Sepharose FF), the extrapolation has been omitted. Lactoferrin bound very tightly to the resins. An isocratic elution could only be performed for S-HyperD and for Fractogel EMD (S) under the applied conditions. As seen in Table 2, lactoferrin could only be eluted at 1 *M* salt.

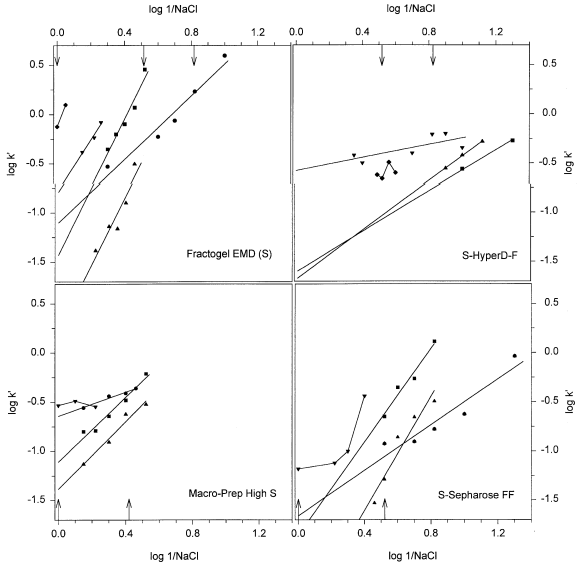

Fig. 8. Summary of isocratic runs with α-lactalbumin, —●—; β-lactoglobulin, —■—; IgG, —▲—; lactoperoxidase, —▼— and lactoferrin, —♦—. The pure proteins were chromatographed on a 10×0.5 cm I.D. column at 150 cm/h. Samples (100 μl) containing 2 mg protein/ml were injected. Arrows indicate the salt concentrations at which stepwise elution was carried out.

Table 3
Dynamic binding capacity for bovine IgG at a linear flow-rate of 100 cm/h

Cation exchanger	Feedstock	
	IgG from whey (mg/ml gel)	Purified IgG (mg/ml gel)
S-HyperD-F	3.3	>20
S-Sepharose FF	3.2	6.6
Fractogel EMD (S)	3.7	>20
Macro-Prep High S	2.1	6.5

4. Discussion

Although a plethora of chromatographic methods have been reported for the fractionation of bovine whey proteins [9–21], cation exchangers with high mechanical stability and improved flow properties have not been investigated for this purpose. S-Sepharose FF, S-HyperD, Macro-Prep High S and Fractogel EMD (S) are used for biopharmaceutical applications [26]. Due to their chemical and physical properties, they are a priori suited for the desired application. They are stable within a pressure drop of at least 3 bar, they can be operated at high flow

velocities with low back pressure and they do not show significant shrinking with salt. The investigated sorbents are either chemically crosslinked Sepharose or completely synthetic media. S-HyperD and Fractogel EMD (S) belong to a completely new generation of chromatographic media. Fractogel, as a member of tentaclegels [27], is considered as a medium with high dynamic capacity.

The media have been tested with the feedstock that is used in industry, presuming acid precipitation of milk is applied. Surprisingly, the dynamic capacities of three media (S-HyperD, S-Sepharose FF and Fractogel EMD S) were in the same range. Macro-Prep exhibited a dynamic capacity that was one third lower (Fig. 5, Table 3). This property of Macro-Prep is explained by the different selectivities that are demonstrated by plots of log k' versus log (1/[NaCl]) (Fig. 8).

As mentioned in Section 1, for industrial applications, the process must be designed so that α-lactalbumin passes straight through the cation exchanger and IgG is adsorbed. After screening with a range of pH values during loading, such conditions were found. The sorbents discriminated between α-lactalbumin and the rest of the proteins present in whey, when the whey was loaded at pH 4.7. For Macro-Prep S, the desired conditions could not be determined. As demonstrated in Fig. 3D, all major whey proteins bind to the cation exchanger and, therefore, the capacity for IgG will be lower than that found in media that do not significantly bind α-lactalbumin.

In all four cases, the dynamic capacity for IgG did not decrease to a large extent with flow-rate, although a range of 100–600 cm/h was investigated (Fig. 5). The capacity was substantially lower when conditions suitable for whey protein fractionation were used. As mentioned in Section 1, conditions had to be found where α-lactalbumin passes through the column and the other proteins can be recovered. Therefore, the running conditions were close to the isoelectric point of bovine IgG. The low capacity can be compensated for by operating the column at high flow-rates and, therefore, high throughput can be obtained. A binding capacity of up to 100 mg bovine IgG/ml gel has been reported for metal chelate interaction chromatography, but the linear flow-rate in these experiments was only 20 cm/h [22]. Cochet et al. [18] obtained a binding capacity of 7.2 mg IgG/ml using immobilized metallized dye chromatography. The linear flow-rate was 20 cm/h. A binding capacity of 12.1 mg bovine IgG/ml at a linear flow-rate of 34 cm/h on thiophilic gel has been reported by Konecney et al [17]. The drawback of these methods, in contrast to the method described here, is the limitation to one individual protein (IgG) in whey. We could combine high selectivity whilst maintaining high throughput.

Fractogel and S-HyperD showed a dynamic capacity of more than 20 mg/ml when purified IgG was applied. Low-molecular-mass compounds in whey, which cannot be removed by ultrafiltration, interfere with the binding of IgG. Interestingly, it is assumed that both sorbents act strongly through surface diffusion [26,28]. These observations indicate that these media do not discriminate between small and large molecules.

Furthermore, different selectivities were observed during elution. The minimal salt concentration at which IgG eluted with the salt front is 0.1 *M* NaCl for S-HyperD and Macro-Prep S. S-Sepharose and Fractogel release IgG with the salt front at 0.3 *M* NaCl. Elution can also be effected at 0.2 *M* NaCl, but the peak volume is very large, a type II elution can be observed [29]. Protein binding to these two sorbents seems to be stronger, since the eluted fractions contain much more lactoglobulin than fractions obtained from S-Hyper D and Macro-Prep S. Lactoferrin can only be completely desorbed using a 1 *M* NaCl buffer. As a result of this binding behaviour, peak volumes from S-Sepharose FF and Fractogel EMD (S) are somewhat larger.

The selectivity data obtained by segmented gradients could be confirmed by isocratic runs using pure whey proteins. At low salt concentrations, the proteins were completely retained on Macro-Prep S. The other three media showed different behaviours, the proteins started to elute at lower salt concentrations. According to the log k'–log (1/[NaCl]) plot, α-lactalbumin passes through the column without significant binding. With S-HyperD, a plot could not be obtained for lactalbumin, since the retention was too weak. Furthermore, a strange plot for S-HyperD was observed, due to the on–off adsorption/desorption behaviour. Complete isocratic elution of the proteins was only possible within a small range.

At low salt concentrations, only a minor portion of the protein was released. The explanation of such elution characteristics may be the very broad charge density distribution, implying a very heterogeneous surface.

When IgG is eluted from S-Sepharose FF, it co-elutes with β-lactoglobulin and α-lactalbumin. Lactoferrin is released at high salt concentrations. This behaviour is again confirmed by results from isocratic runs (compare Table 2, Fig. 8). Furthermore, the potent retention behaviour of Fractogel is validated by isocratic runs, since plots are shifted towards higher salt concentrations. The slope of the approximated function gives a measure for the strength of the interaction of the protein with the surface of the ion exchanger [25]. Proteins showed the biggest slope when chromatographed on Fractogel.

Due to the high throughput, the selectivity and the simple buffers, the investigated cation exchangers are suitable for large scale fractionation of bovine whey. Depending on the desired purpose, one of these materials can be chosen. A second purification step must be added, if high purity of a certain protein is desired. In the current work, the emphasis was put on selectivity. For economic reasons, the overloaded situation has to be studied and certain displacement effects under these conditions may alter the picture obtained.

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