Isolation and purification of immunoglobulin G from bovine colostrums by hydrophobic charge-induction chromatography

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ABSTRACT

Hydrophobic charge-induction chromatography, a novel chromatographic technique for bioseparation, was developed to isolate and purify bovine IgG with high purity. In this work, the raw IgG solution, a precipitate from bovine colostrum powder solution with 40% (wt/vol) ammonium sulfate, was dissolved in 50 mM phosphate buffer and used as loading solution for investigating chromatographic conditions on a mercapto-ethyl-pyridine (MEP) HyperCel (Pall Corp., Port Washington, NY) sorbent. The initial IgG concentration had no effect on the dynamic binding capacity of MEP HyperCel resin, but the linear velocity of loading solution had an obvious effect on the dynamic IgG binding capacity and IgG recovery. The maximum linear velocity was optimized as 0.4 cm/min of loading solution, and 90% recovery of IgG was achieved. Under these optimized binding conditions, the pH and ionic strength for the elution process were selected as pH 4.5 and 0.5 M NaCl, respectively. Subsequently, hydrophobic charge-induction chromatography was performed on a MEP HyperCel sorbent to isolate IgG using bovine colostrum whey as the loading solution. Under the optimized operation conditions, a remarkable process improvement in IgG purification was received, which includes a yield of 91.5%, a purity of 93.9% (wt/wt), and a purification factor of 6.8. The results indicated that MEP HyperCel chromatography offers an efficient means to purify IgG from bovine colostrums.

Key words: immunoglobulin G, mercapto-ethyl-pyridine HyperCel, bovine colostrums, hydrophobic charge-induction chromatography

INTRODUCTION

As a major whey protein in bovine colostrum and milk, IgG has several biological functions, including

improving immunity and antibacterial properties (Korhonen et al., 1998; Uruakpa et al., 2002; Madureira et al., 2007; Indyk et al., 2008). Immunoglobulin G from bovine colostrum has been used in the food industry in many countries, such as New Zealand, Australia, and France. With the increasing demand of IgG, it is necessary to develop a purification process with high purity, high yield, and low cost. Some traditional separation methods have been routinely used in IgG isolation and purification, such as reverse micelles (Su and Chiang, 2003), ion-exchange chromatography (Hahn et al., 1998; Bai et al., 2000; Doultani et al., 2004; Wongchuphan et al., 2011), and affinity chromatography (Bottomley et al., 1995; Kim and Li-Chan, 1998; Yang et al., 2009; Barroso et al., 2010). In recent years, serial chromatographic techniques were developed to separate bovine IgG; for example, Qi et al. (2001) developed an HPLC method that was based on the coupled diethylamino-ethanol (DEAE) anion-exchange and protein G affinity columns for isolation of IgG, and Wu and Xu (2009) purified IgG from bovine colostrum with serial cation-anion exchange chromatography. These works showed that serial chromatography is useful to separate bovine IgG with relatively high purity and high efficiency, but some operating conditions of isolation process are strictly controlled, such as initial protein concentration and operating temperature, or they have limitations, such as low recovery of IgG, and high costs for the isolation process.

More recently, hydrophobic charge-induction chromatography (HCIC), a novel chromatographic technique for bioseparation, was developed for the purification of active proteins (Yang and Geng, 2011). This technique is based on the pH-dependent behavior of ionizable, dual-mode ligands. Adsorption is based on mild hydrophobic interaction and is achieved without addition of lyotropic or other salts. Desorption is based on charge repulsion and performed by reducing pH. The HCIC mixed-mode chromatography has many advantages over conventional chromatography, such as high resolution, high selectivity, high sample loading, and especially

Received November 23, 2014.

Accepted January 24, 2015.

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the ability to replace 2 conventionally corresponding columns. It brings a specific mixed-mode or multimodal separation mechanism, different from conventional ion exchange or affinity mechanisms, and is particularly effective to replace conventional hydrophobic induction chromatography (HIC) for direct capture of IgG from various feedstocks. Due to its immunoglobulin-selective nature and more than one form of interaction between the stationary phase and the solutes in a feed stream, HCIC chromatography probably overcomes the problems of large-scale purification of IgG with conventional ion exchange chromatography (Zhao et al., 2009). Mercapto-ethyl-pyridine (MEP) HyperCel resin (Pall Corp., Port Washington, NY) is a mixed-mode resin based on the mechanism of HCIC (Chen et al., 2010) that has been used to purify the monoclonal antibodies against botulinum neurotoxin serotype A (Mowry et al., 2004).

In the current study, MEP HyperCel mixed-mode chromatography was developed to isolate and purify bovine IgG with high purity. The optimization of operation conditions both for the adsorption process and the elution process were investigated. The recovery and purification fold were higher than other chromatography methods with the reduction of salt concentration (Wongchuphan et al., 2011).

MATERIALS AND METHODS

Materials

Bovine colostrum powder was purchased from Kanpure Corporation (Heilongjiang, China). Bovine colostrums were obtained from Xiaoshan dairy farm (Hangzhou, China). The mixed-mode resin used in this study was HCIC resin; MEP HyperCel was purchased from Pall Corporation. Bovine IgG standard sample was purchased from Sigma (St. Louis, MO). Hi-Trap Protein G column was purchased from GE Healthcare (Piscataway, NJ). Electrophoresis kits were purchased from Bio-Rad (Hercules, CA). Deionized water used for buffer solutions was prepared by water purification system from Sartorius (Gottingen, Germany). All chemical reagents were analytical grade. The ÄKTA explorer 100 systems were purchased from GE Healthcare (Uppsala, Sweden).

Sample Pretreatment

Bovine colostrum powder was dissolved in 300 mL of deionized water, and 2 M HCl was applied to adjust the pH value to 4.6. After resting for 20 min, the solution was centrifuged in a Sigma MA3-18 centrifuge (Osterode am Harz, Germany) at $3,960 \times g$ for 20 min at

4°C; then IgG was precipitated with ammonium sulfate (40%, wt/vol) from the supernatant. After centrifugation was performed in the same way, the obtained precipitate was dissolved in equilibration buffer (50 mM phosphate buffer, pH 6–9) labeled “primary sample 1,” which was used as the loading solution for optimization of the isolation process. The IgG purity determined by affinity chromatography using Hi-Trap Protein G column was 80% (wt/vol).

Skimmed colostrum were prepared from bovine colostrum by centrifugation in a Sigma MA3-18 centrifuge at $3,960 \times g$ for 20 min at 4°C. After delipidation, the pH value of the skimmed colostrum was adjusted to 4.6 with 2 M HCl. After resting for 30 min, the solution was centrifuged under the same condition as primary sample 1 and filtered by 8-layer gauze in series. The supernatant with 13.8% (wt/wt) purity for IgG determined by affinity chromatography as described previously was labeled “primary sample 2” and stored at -20°C .

Adsorption Experiments

The adsorption experiments of all chromatographic separations were performed on an ÄKTA explorer 100 system. Primary sample 1, at different pH or ionic strengths, was loaded to the pre-equilibrated column ($\Phi 1.6 \times 7$ cm) packed with MEP HyperCel resin. The solution flowed through the column at a certain rate and was collected every other minute.

Desorption and Regeneration

In the processes of all chromatographic separations, phosphate buffer at pH 7.0 was applied to wash off the unabsorbed whey protein first after breakthrough of the loading solution. Then, deionized water or sodium acetate buffers at different pH and concentrations were used to elute the columns one after another at a flow rate of 100 cm/h. After each run, the column was cleaned with 1 M NaOH. Each chromatographic separation was operated under the conditions described in detail in the results or in figure legend.

Hi-Trap Protein G Column HPLC

An HPLC system was used for determining the purity of all samples and protein fractions. It consists of a Laballiance Serial III (State College, PA) interfaced with a model 500 absorbance detector, a JS-3050 data acquisition, and a manipulation system. A 1-mL Hi-Trap Protein G column was used with a flow-rate of 0.4 mL/min at 20°C in this HPLC assay, and gradient elution was carried out with 2 solvents. Solvent I was 0.05

M sodium phosphate buffer at pH 6.5 and solvent II was 0.05 M glycine-HCl buffer at pH 2.5. All buffer solutions and samples were degassed after filtration through a 0.45- μ m cellulose acetate membrane. The column was equilibrated with 100% solvent I. After a 20- μ L sample was injected, a 1-min isocratic period was followed by a 20-min linear gradient to 0% solvent I (100% solvent II). One-minute linear gradient to 100% solvent I was applied after 1 min with 0% solvent I. The column was re-equilibrated with 100% solvent I for 5 min. Detection wavelength was set at 280 nm. A 6-point standard curve was constructed from the IgG standard solutions at working concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL, respectively. Prior to Hi-Trap Protein G column-HPLC analysis, samples of the colostrum and elution fractions were filtered through 0.45 μ m of cellulose acetate membrane. The IgG concentration of all samples was adjusted to bring the likely range of the standard curve before determined.

SDS-PAGE

Desalted elution fractions were analyzed by SDS-PAGE (Su et al., 2003). Standards solutions and eluent samples were diluted in sample buffer to 1 mg/mL and heated in water at 95°C for 15 min. Load volume of samples was 20 μ L. The voltage of 80 V was used at the beginning of electrophoresis, and the voltage was switched to 110 V after the sample ran into the separating gel. Protein bands on gels stained with Coomassie Brilliant Blue were imaged by Quantity One (Bio Rad; Wang et al., 2011).

Dynamic Binding Capacity

All samples were loaded onto the chromatographic column. The concentrations of IgG in the solutions collected from the effluent every other minute were determined by affinity HPLC until the absorbance at 280 nm had only a slight variation. Then, the breakthrough curve for the absorption process was plotted. The binding capacity at 10% breakthrough was defined as the dynamic binding capacity of MEP HyperCel resin calculated with the following equation:

$$q_{10\%} = \frac{C_0 \times \int_0^{V_{10\%}} \left(1 - \frac{C}{C_0}\right) dV}{M},$$

where C is the concentration of IgG in the flow-through (mg/mL); C_0 is the concentration of IgG in the primary sample (mg/mL); V is the volume of the loading solution (mL); dV is differential volume of the loading

solution (mL); M is the total mass of MEP HyperCel resin (g); $q_{10\%}$ is the binding capacity of 1 g of MEP HyperCel resin at 10% breakthrough (mg/g of resin); $V_{10\%}$ is the volume of the loading solution at 10% breakthrough (mL).

Calculations

The purity of IgG is defined as the ratio of the amount of IgG in protein fractions to the total amount of proteins in bovine whey (Wongchuphan et al., 2011):

$$\text{Purity} = \frac{\text{Amount of IgG in protein fractions}}{\text{Total amount of proteins in bovine whey}}.$$

Yield is expressed as the percentage of the amount of IgG in protein fractions divided by the total amount of IgG in bovine whey:

$$\text{Yield} = \frac{\text{Amount of IgG in protein fractions}}{\text{Total amount of IgG in bovine whey}} \times 100\%.$$

The purification factor is calculated from the purity of IgG in the protein fractions divided by the purity of IgG in bovine whey:

$$\text{Purification factor} = \frac{\text{Purity of IgG in protein fractions}}{\text{Purity of IgG in bovine whey}}.$$

RESULTS AND DISCUSSION

Optimization of Adsorption Process

Effect of Initial IgG Concentration on Dynamic Binding Capacity of MEP HyperCel Resin.

The concentration of IgG and its percentage in fresh bovine colostrum are quite different, with factors such as the different stages in milk production, the different types of dairy cows, and individual difference (Leary et al., 1982; Mee et al., 1996; Nielsen et al., 1996; Wouters, 2012). Correspondingly, the content of IgG in colostrum would change a lot on the basis that IgG was the main ingredient of bovine immunoglobulins (Pakkanen and Aalto, 1997). To evaluate the effect of different types of bovine colostrum on IgG adsorption capacity for MEP HyperCel resin, the primary samples with different concentrations of IgG were used to determine the dynamic binding capacity of the novel mixed-mode resin. For evaluating the performance of MEP HyperCel resin under the relatively high-speed velocity, the initial concentrations of IgG selected were 0.5, 1.0, 2.0, 4.0, and 6.0 mg/mL with the linear velocity of 1.6 cm/min.

Figure 1. Mercapto-ethyl-pyridine (MEP) HyperCel (Pall Corp., Port Washington, NY) chromatogram of primary sample 1. Peak A represents the process of loading the primary sample 1 and washing off the unabsorbed protein; peak B represents the process of eluting the absorbed protein; Cond = conductivity of loading solution. Color version available online.

Section A in Figure 1 shows the process by which primary sample 1 flowed through the column. Primary sample 1 was loaded to the MEP HyperCel column and the unabsorbed proteins were washed off with the phosphate buffer. Section B shows the process by which the absorbed protein was eluted by 200 mM sodium acetate buffer at pH 3.0 directly.

As shown in Figure 2, it was clear that the binding capacity of MEP HyperCel resin for IgG at 10% breakthrough changed irregularly from 9.6 to 10.4 mg/g. As the concentration of IgG decreased from 6 to 0.5 mg/mL, the dynamic binding capacity of MEP HyperCel resin fell slightly. On the whole, in this concentration range, the concentration of IgG had no effect on the binding capacity of MEP HyperCel resin, and it's unnecessary to concentrate the primary sample used in an adsorption process with a lower concentration of IgG.

Effect of Linear Velocity on Dynamic Binding Capacity of MEP HyperCel Resin. Linear velocity has a significant effect on the quality of products, yield, and time of the separation process. To enlarge the scale and shorten the time of the production cycle, the correlation among the recovery, binding capacity, and linear velocity were studied to obtain the best balance point between the production cycle and yield. As a result of the relatively low level of dynamic binding capacity of IgG obtained at the high linear velocity of 1.6 cm/min (Figure 2), we chose the linear velocities of 0.3, 0.4, 0.6, and 1.2 cm/min with the IgG concentration of 6.0 mg/mL in the current experiment.

Figure 3 indicated that the maximum linear velocity was 0.4 cm/min for loading when the recovery of IgG

achieved 90%, and the different linear velocity had an effect on the dynamic binding capacity for IgG. When the linear velocity for loading was too high, the binding capacity at 10% breakthrough was very low; the binding capacity (18.1 mg/g of resin) at the linear velocity of 0.4 cm/min was much higher than that (11.3 mg/g of resin) at the linear velocity of 1.2 cm/min. Therefore, the linear velocity for the primary sample was selected as 0.4 cm/min.

Optimization of Elution Process

pH Gradient Elution. The adsorption mechanism of MEP HyperCel resin for IgG was based on the fact ionizable ligands (MEP) had a dependency on pH. To determine the optimum pH for elution during the isolation process, gradient elution was performed with mobile phase A (0.3 M HCl) and mobile phase B (0.2 M sodium acetate buffer at pH 6.5).

We found that the pH of the solution that flowed out from the column did not decrease as the volume percentage of HCl solution in eluent increased from 0% in section A of Figure 4. This phenomenon indicated that the change of pH had a lag, because some sodium acetate buffer at pH 6.5 was observed in the column. In section B of Figure 4, as the pH value declined, IgG was desorbed from MEP HyperCel resin in the isolation process; likewise, the UV absorption value at 280 nm of the solution that flew out from the column reached the maximum at pH 4.5, which declared that the optimum pH for elution was pH 4.5. Furthermore, pH decreased slowly before the elution volume reached 150 mL and then remained constant. Another peak arose when the elution volume reached 150 mL; the reason for this was

Figure 2. Effect of IgG concentration on dynamic binding capacity of mercapto-ethyl-pyridine (MEP) HyperCel (Pall Corp., Port Washington, NY) chromatography.

Figure 3. Effects of linear velocity on dynamic binding capacity (□) and recovery (■) of mercapto-ethyl-pyridine (MEP) HyperCel (Pall Corp., Port Washington, NY) chromatography. Color version available online.

that the primary condition maintained by the buffer solution in the column was broken down by the eluent containing 20% sodium acetate buffer and 80% HCl, thus making the pH decline suddenly according to the pH curve in section C of Figure 4 and all residual proteins were washed off.

Determination of the Initial Elution Parameters for Isolation of IgG from Bovine Whey with MEP HyperCel Resin. Primary sample 2 (bovine

colostrum whey) was loaded to the pre-equilibrated column packed with MEP HyperCel resin, and the whole process of the isolation is shown in Figure 5. We found that an absorption peak of proteins came out due to the difference in ionic strength during the elution with the deionized water, and most proteins were washed off with 50 mM sodium acetate buffer at pH 4.5; then, 200 mM sodium acetate buffer at pH 3.0 desorbed the rest of proteins.

Protein fractions from chromatographic elutions were analyzed by SDS-PAGE. In Figure 6, lane 5 shows that the loose IgG bonded to MEP HyperCel resin and a minority of small molecular proteins with the molecular weight between 17 and 28 kDa were washed off with de-ionized water; lane 6 showed that the chief component in protein fraction from elution with 50 mM sodium acetate buffer at pH 4.5 was IgG, which did not have too high a purity for IgG caused by the large number of small molecular proteins. Then, lane 7 shows that there was no IgG in the protein fraction from elution with 200 mM sodium acetate buffer at pH 3.0, which was consistent with the consequence of the pH gradient elution. Furthermore, it was found that the proteins with the molecular weight between 17 and 36 kDa could integrate with MEP HyperCel resin. Taking the effect of small molecular proteins on the purity of IgG into account, a further optimization was essential for

Figure 4. pH gradient elution profile on mercapto-ethyl-pyridine (MEP) HyperCel (Pall Corp., Port Washington, NY) chromatography. A, B, and C represent elution volumes from 0 to 50 mL, from 50 to 150 mL, and >150 mL, respectively. Color version available online.

Figure 5. Mercapto-ethyl-pyridine (MEP) HyperCel (Pall Corp., Port Washington, NY) chromatography of bovine whey. Peak A represents load and elution by 50 mM phosphate buffer at pH 7.0; peak B is deionized water; peaks C and D denote elution by sodium acetate buffers with different pH; Cond = conductivity of the loading solution. Color version available online.

the elution process for the chromatography based on MEP HyperCel resin.

Optimization of the Purification Process of IgG Based on HCIC with MEP HyperCel Resin. Immunoglobulin G absorbed on the MEP HyperCel resin in the neutral condition could be desorbed by electrostatic interactions. When the pH of the buffer solution decreased, the strength of charge depended on the isoelectric point of proteins in bovine whey and the pKa of the ligands. Based on the difference between the pH gradient elution and the hydrophobicity of different proteins in bovine whey, the effect of the pH and the ionic strength of the buffer solution on the purification process of IgG was investigated.

The purification process of IgG based on HCIC was performed by pH gradient elution, with 0.3 M HCl as the mobile phase A and 0.2 M sodium acetate buffer at pH 6.5 added by 0.25 M, 0.5 M, and 1.0 M NaCl in stepwise manner as the mobile phase B. The result of isolation of bovine colostrum whey by MEP HyperCel chromatography is shown in Figure 7.

Section A in Figure 7 shows during the process in which primary sample 2 flew through the column; primary sample 2 was loaded on the MEP HyperCel column and the unabsorbed protein were washed off with the phosphate buffer. Sections B, C, D, and E in

Figure 6. The SDS-PAGE profile of bovine whey and fractions from mercapto-ethyl-pyridine (MEP) HyperCel (Pall Corp., Port Washington, NY) chromatography. Lane M = standard protein markers; lane 1 = IgG; lane 2 = IgA; lane 3 = acid whey; lane 4 = sample from precipitation of 40% $(\text{NH}_4)_2\text{SO}_4$; lanes 5, 6, and 7 = fractions eluted by different pH and concentration of sodium acetate buffer. Color version available online.

Figure 7. Elution profile of IgG on mercapto-ethyl-pyridine (MEP) HyperCel (Pall Corp., Port Washington, NY) chromatography by sodium acetate buffers with different pH and ionic strength. Peak A represents load and elution by deionized water; peaks B, C, D, and E denote elution by sodium acetate buffers with different pH and ionic strength. Color version available online.

Figure 7 show that the absorbed protein washed off from the MEP HyperCel column one by one using pH gradient solution with 50 mM sodium acetate buffer added to 0.5 M NaCl at pH 5.5, 4.5, and 4.0, and 200 mM sodium acetate buffer at pH 3.0 in stepwise manner.

Protein fractions from chromatographic elution were analyzed by SDS-PAGE. In Figure 8, lane 3 shows that the elution peak contained a lot of small molecular proteins and very little IgG; there was no band for impurity proteins in lane 4 and the high purity of IgG was confirmed by affinity chromatography. Furthermore, lane 5 showed that the corresponding elution peak contained few impurity proteins bonded with MEP HyperCel resin with the molecular weight between 17 and 36 kDa. In addition, no clear band for proteins appeared in lane 6, meaning no proteins or very little proteins were observed in this elution peak; therefore, it is not necessary to elute the column with 200 mM sodium acetate buffer at pH 3.0 and whey proteins absorbed by MEP HyperCel resin could be washed off completely with sodium acetate buffer at pH 4.0.

Under this optimized condition of MEP HyperCel chromatography, we recorded the results that were showed in Table 1. The yield of IgG was 91.5%, obviously higher than the yield from the serial cation-anion

exchange chromatography by Wu and Xu (2009). The special mechanism for isolation and purification of IgG with MEP HyperCel resin resulted in the high purity of

Figure 8. The SDS-PAGE profile of bovine whey and fractions from mercapto-ethyl-pyridine (MEP) HyperCel (Pall Corp., Port Washington, NY) chromatography. Lane M = standard protein markers; lane 1 = IgG; lane 2 = bovine whey; lanes 3, 4, 5, and 6 = fractions eluted by sodium acetate buffers with different pH (5.5, 4.5, 4.0) and ionic strength (200 mM). Color version available online.

Table 1. Purification of IgG from bovine whey by optimized mercapto-ethyl-pyridine (MEP) HyperCel (Pall Corp., Port Washington, NY) chromatography

Sample	Purity (%)	Purification factor	Recovery (%)
Bovine colostrum whey	13.8	1	100
Eluent from MEP HyperCel chromatography	93.9	6.8	91.5

IgG from the process; the purity of IgG was 93.9% and the recovery exceeded 90.0%. Therefore, it is appropriate for industrial applications.

CONCLUSIONS

In this work, primary sample 1, which was prepared from bovine colostrum powder, was used as the loading solution for the optimization of the purification process because it had a relatively high purity determined by affinity HPLC. The results from the optimization of the adsorption process were that the optimum linear velocity was 0.4 cm/min and the initial IgG concentration had no effect on the dynamic binding capacity. Under these optimal conditions, the optimization of the purification for bovine colostrum whey based on the mixed-mode chromatography with MEP HyperCel resin was investigated. This experiment is the first to use HCIC to isolate and purify the IgG from bovine colostrum. By comparing the results before and after the optimization, yield, recovery, and purity for IgG were improved significantly. Normally, high-purity IgG is isolated and purified by affinity chromatography, but affinity chromatography is not generally used for commercial production of food protein ingredients due to the high cost. The MEP HyperCel resin has a strong affinity for IgG under the optimized operating conditions at relatively high velocity and is a potential replacement for protein A and protein G affinity resin. In addition, the HCIC process shows significant benefits, including easy whey pretreatment without any adjustment of pH or ionic strength, high purity achieved in a single step, high IgG capacity, and relatively low cost. Therefore, the HCIC process has the potential ability to be scaled up to commercial-scale production of high-purity bovine IgG from various feedstocks.

ACKNOWLEDGMENTS

The authors are grateful for the financial support from the National Natural Science Foundation of China, Beijing, China (No. 21376215), the Science and Technology Project of Zhejiang Province, Hangzhou, China (2010C33122), the National Science and Technology Major Project of New Drug, Beijing, China (No. 2012ZX09103101-075), the Major State Basic

Research Development Program of China, Beijing, China (No. 2011CB710803), the National High-Tech Research and Development Program of China, Beijing, China (2012AA022302), and the Innovative Research Platform co-constructed by Zhejiang University and Taizhou City, Taizhou, China.

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