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Performance of hexamer peptide ligands for affinity purification of immunoglobulin G from commercial cell culture media☆

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ABSTRACT

Previous work has reported on the identification and characterization of the hexapeptide ligands HWRGWV, HYFKFD, and HFRRHL for the affinity capture of IgG through specific binding to its Fc fragment. This paper addresses issues related to the successful application of these ligands, on a commercial methacrylate chromatographic resin, for the purification of IgG from mammalian cell culture fluids. The concentrations of sodium chloride and sodium caprylate in the binding buffer were optimized to maximize the purity and yield of IgG upon elution. Screening of several regeneration conditions found that either 2 M guanidine–HCl or a combination of 0.85% phosphoric acid followed by 2 M urea resulted in complete recovery of the IgG adsorption capacity and that the column could be reused over many cycles. The hexapeptide ligands were used for the purification of humanized and chimeric monoclonal antibodies from two commercial CHO cell culture fluids. The chimeric MAb of IgG1 subclass was purified using the HWRGWV resin whereas the humanized MAb of IgG4 subclass was purified using the HWRGWV, HYFKFD and HFRRHL resins. The purities and yields obtained for both the MAbs were found to be higher than 94% and 85% respectively. These results compare well with the yields and purities obtained using Protein G columns. The residual DNA and host cell protein reduction obtained by the HWRGWV resin was in the range of 4 log reduction value (LRV) and 2 LRV respectively, comparable to those reported for Protein A resins. The dynamic binding capacity of all three peptide resins for the humanized monoclonal antibody was in the range of 20 mg/mL.

1. Introduction

Monoclonal antibodies (MAbs) and Fc-fusion proteins had annual sales of $34 billion in 2008 [1]. At that time, MAbs and Fc-fusion proteins accounted for 275 of the biotherapeutic products in the FDA pipeline in various stages of clinical trials. These important biologics have transformed the treatment of many cancers, autoimmune and infectious diseases and they offer tremendous potential to treat a wide range of other acute conditions. However, MAb-based therapies are very expensive, with costs of several thousand dollars per dose, making them unaffordable to many patients. With growing competition from follow-on biologics and increased pressure from the high cost of healthcare, the success of next generation MAbs may be largely governed by the economic factors [2,3].

Progress in mammalian cell culture and fermentation technologies have enhanced antibody productivity from milligrams to grams per litre with titres >5 g/L becoming increasingly common [4,5]. However, downstream processing technology, which accounts for anywhere between 50 and 80% of total production costs [6], has not kept up with advances in upstream processes. A major contributor to downstream processing costs of antibodies is the use of Protein A or Protein G affinity product capture steps following cell removal. Since Protein A and G bind very strongly to antibodies, product elution is normally done at pH 3, which can result in antibody aggregation, while also serving as a viral inactivation step. Repeated cycles of binding and elution, together with periodic cleaning and sanitization of the resin using 0.1–0.5N NaOH, can result in loss of activity of the Protein A and Protein G chromatographic supports as a result of denaturation of the three-dimensional tertiary structures of these ligands. Even though engineered versions of Protein A and Protein G for greater stability are available [7,8], they are also more expensive. To address these issues, there has been great interest over the years, on the part of both industry and academia, on the discovery and validation of more efficient and less costly affinity ligands for antibodies. Among the various types of alternative ligands developed, small synthetic ligands have received a lot of attention due to their higher stability and lower cost relative to large protein ligands. Several such synthetic ligands have been developed for antibody purification [9–23]. Nevertheless, these small ligands have not

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made huge inroads into the market for Protein A, mostly due to their lack of specificity. Resins with larger ligands, such as camelid antibodies exhibit excellent specificity but can be as expensive as Protein A and G.

Our research group has identified peptide ligands from combinatorial solid-phase hexapeptide libraries for the purification of various biomolecules such as α-1-proteinase inhibitor [24], α-lactalbumin [25], staphylococcal enterotoxin B [26–28] and fibrinogen [29]. Three hexapeptide ligands that bind to the Fc fragment of hIgG, HWRGWV, HYFKFD, and HFRRHL, have been identified and preliminary comparisons of their binding properties have been made to both Protein A and the small triazine-based ligand MAbsorbent A2P [30,31]. Mass spectrometry analysis and molecular docking calculations have been used to determine the putative binding site of HWRGWV on the Fc portion of hIgG [32]. These small peptide ligands exhibit higher specificity than small organic ligands, but lower binding avidity than Protein A and Protein G. They have the advantage of being able to be synthesized chemically under cGMP conditions, offering the potential for lower production costs. Most of the work done on these peptide ligands has involved chromatographic resins on which the peptide ligand is synthesized directly on the chromatographic support using standard solid phase peptide synthesis techniques. This direct synthesis method may offer significant cost advantages over the more common practice of synthesizing pure peptide ligand, followed by attachment to the chromatographic resin from a suitable solvent at a prescribed surface density.

During experiments on the capture of IgG by the HWRGWV resin from complete minimal essential medium (cMEM) containing 10% fetal calf serum it was found that there was some contamination of the product by bovine serum albumin (BSA) [31]. This nonspecific interaction of BSA to the peptide resin is most likely electrostatic in nature and is believed to be due to un-reacted groups and/or truncated peptides which are formed during the solid phase peptide synthesis of the peptide on polymethacrylate-based Toyopearl Amino 650 M resin. In order to prevent nonspecific BSA binding, the effects of NaCl and sodium caprylate in the equilibration and binding buffers were studied. The resulting yield and purity of IgG under these conditions are presented in this work. In addition, results are presented on the identification of suitable regeneration agents for retaining the performance of the peptide ligand column over many cycles. Regeneration conditions for affinity resins are highly dependent on the nature of the ligand and the support, as well as on the type of impurities that are to bind strongly to the column [33]. The ability to regenerate and clean affinity supports is an important consideration for industrial applications.

The present work also reports on the efficacy of the peptide resins in purifying humanized and chimeric monoclonal antibodies from Chinese Hamster Ovary (CHO) cell culture fluids. Both of these MAbs are commercial products in their clarified cell culture fluids. The chimeric MAb of IgG1 subclass was purified using the HWRGWV resin whereas the humanized MAb of IgG4 subclass was purified using HWRGWV, HYFKFD and HFRRHL resins. Results are presented on the dynamic binding capacity, yield and purity of MAbs obtained during the peptide affinity capture step, as well as on the ability of the HWRGWV peptide resin to effectively remove residual DNA and host cell proteins (HCPs). The results are compared to those found using the industry's standard Protein A and G columns.

2. Experimental

2.1. Materials

HWRGWV, HYFKFD, and HFRRHL resins with a peptide density of 0.15 mequiv./g were obtained from CreoSalus (KY, USA). The peptide was synthesized directly on Toyopearl AF-Amino-650 M (particle size 65 μm, Tosoh Bioscience, Montgomeryville, USA) using the fluorenylmethyloxycarbonyl (Fmoc) coupling chemistry. Human polyclonal immunoglobulin G (IgG) in lyophillized form was purchased from Equitech-Bio, Inc. (TX, USA). Sodium chloride, sodium acetate, sodium thiocyanate, ethylene glycol, glycine, guanidine–HCl, urea, hydrochloric acid, glacial acetic acid, phosphoric acid were obtained from Fischer Scientific (PA, USA). Phosphate buffer saline at pH 7.4, ethanol, methanol and isopropanol were from Sigma (MO, USA). All the solvents were of analytical grade. Cell culture medium (Eagle Minimum Essential medium, EMEM) was from Quality Biological (MD, USA). Fetal calf serum (FCS) and tryptose phosphate broth (TPB) were obtained from Hyclone (UT, USA) and Becton Dickinson (MD, USA), respectively.

The complete mammalian cell culture medium (cMEM) used for NaCl, sodium caprylate and regeneration studies was prepared by combining EMEM with 10% FCS and 5% TBP. For the purification experiments, two CHO cell culture supernatants containing monoclonal antibody (MAb) were obtained from two different biopharmaceutical manufacturers that asked for confidentiality. The monoclonal antibodies, MAb1 and MAb2 belong to different IgG classes. MAb1 is a humanized IgG4 whereas MAb2 is a chimeric IgG1. The concentration of MAb1 in the supernatant was 1.5 mg/mL and the concentration of MAb2 in its supernatant was 2.3 mg/mL. In addition to the MAbs, these supernatants contained the usual set of impurities found in mammalian cell culture fluids: pluronic acid, yeast hydrolysate, cholesterol, tropolone, amino acids, glucose, vitamins and cellular components.

Micro BCA assay kits were purchased from Pierce (IL, USA). NuPAGE® Novex gels (4–12% Bis-Tris), NuPAGE® MOPS and MES running buffers, NuPAGE® LDS sample buffer, NuPAGE® reducing agent, SeeBlue plus2® pre-stained molecular weight marker, SimpleBlue™ SafeStain were all from Invitrogen (CA, USA). A HiTrap™ Protein G column was purchased from GE Healthcare (NJ, USA). A Waters 626 LC system integrated with 2487 UV detector (Waters, MA, USA) was used for all chromatography runs unless otherwise mentioned. Microbore stainless steel columns with dimensions 30 mm long × 2.1 mm I.D. were from Altech-Applied Science (PA, USA). All experiments were carried out at room temperature.

2.2. Influence of NaCl concentration on IgG yield and purity

Thirty five milligrams of HWRGWV resin were dry-packed in 30 mm × 2.1 mm I.D. microbore columns (0.1 mL). The resin was swollen with 20% methanol and then washed with phosphate buffered saline (PBS), pH 7.4. A sample solution of IgG was prepared by spiking 10 mg of IgG into 1 mL of cMEM. The effect of salt concentration in the equilibration and binding buffers was studied at 0.25 M, 0.5 M, 0.75 M and 1 M NaCl. NaCl concentrations listed are in addition to the salts already present in PBS (0.14 M NaCl). The NaCl concentration in the binding buffer was adjusted to the desired concentration listed above. After equilibrating the column with the binding buffer, 100 μL of feed was loaded onto the column at a flow rate of 0.05 mL/min (87 cm/h). The column was washed with 4 mL of binding buffer at flow rate of 0.4 mL/min (692 cm/h). Elution was then performed with 4 mL of 0.2 M acetate buffer, pH 4 at a flow rate of 0.4 mL/min. The effluent was monitored by absorbance at 280 nm. Fractions were collected and concentrated five times by centrifugation at 4 °C, 20817 × *g* for 30 min using an Amicon Ultra centrifugal filter (3000 MWCO, Ultracel®, Millipore, MA, USA). These fractions were then used for analysis of IgG purity and yield.

2.3. Influence of sodium caprylate on IgG yield and purity

The HWRGWV column was packed and swollen as described in Section 2.2. A sample solution of IgG was prepared by spiking 10 mg of IgG into 1 mL of cMEM. The effect of sodium caprylate in the binding buffer was studied at different concentrations of sodium caprylate *viz*. 0 mM, 25 mM, 50 mM and 75 mM. The sodium caprylate concentrations in the binding buffer and in the feed mixture were adjusted to the above listed concentrations in a base buffer of PBS containing 0.25 M NaCl. The chromatographic steps, fraction collection and preparation were same as described in Section 2.2.

2.4. Sample analysis for yields and purities

The amounts of IgG in the collected fractions were quantified by HPLC using a 1-mL HiTrap Protein G column. The yield of IgG was calculated as the ratio of IgG eluted to total IgG loaded. The purity of IgG in the eluted fractions was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing or non-reducing conditions, as described by Yang et al. [30], using NuPAGE® Novex 4–12% Bis-Tris gels in a Xcell SuperLock™ Mini-Cell system (Invitrogen). Sample preparation was done by adding 5 μL of NuPAGE® LDS buffer and 2 μL of NuPAGE® reducing agent to 13 μL of sample and boiling the resulting mixture for 10 min. Gels were Coomassie-stained by using SimpleBlue™ SafeStain. The IgG purity was determined by densitometric analysis of Coomassie-stained gels by means of ImageJ 1.32j software (National Institutes of Health, MD, USA). The purity of the product was calculated as the fraction of the total area equivalent to the IgG bands at 25 and 50 kDa.

2.5. Batch screening of various regeneration solutions

The following regeneration agents at different concentrations were selected for the screening study: sodium thiocyanate (1 M, 2 M, 3 M), urea (2 M, 5 M, 8 M), guanidine-HCl (1 M, 2 M, 6 M), 0.1 M glycine pH 2.5, 0.85% (v/v) phosphoric acid, 0.85% phosphoric acid followed by 2 M Urea in sodium acetate pH 4 buffer, 10% (v/v) ethanol, 10% (v/v) isopropanol, 50% (w/w) ethylene glycol. Fourteen aliquots of 10 mg of HWRGWV resin were swollen in 20% (v/v) methanol for 30 min. After equilibration with PBS pH 7.4, each aliquot was incubated in 1 mL of cMEM mixture containing 10 mg/mL IgG for 2 h. Resins were thoroughly washed in PBS to remove the unbound proteins and then incubated for 1 hour in 1.5 mL of regeneration solution. The supernatants were collected and analyzed for the total protein content by bicinchoninic acid (BCA) method. The regeneration efficiency was calculated as ratio of protein desorbed by the regeneration agent to the total protein loaded on the resin. To further test the regeneration, 20 μL of regenerated resins were washed with PBS, boiled with 20 μL SDS buffer (mixture of 25% NuPAGE® LDS buffer and 10% NuPAGE® reducing agent) for 10 minutes and the resulting supernatants were analyzed by SDS-PAGE.

2.6. Secondary screening of the selected regeneration agent

The most efficient regeneration agents were further tested in sequential cycles of chromatographic runs. HWRGWV resin was dry-packed in 0.1 mL column, swollen and washed with PBS as described in section 2.2. The resin was equilibrated with PBS containing 1 M NaCl. A sample of 100 μL of cMEM containing 10 mg/mL IgG was loaded onto the column in the equilibration buffer at a flow rate of 0.05 mL/min (87 cm/h). The column was washed with 4 mL of binding buffer at a flow rate of 0.4 mL/min (692 cm/h). Elution and regeneration were then performed simultaneously with 4 mL of selected regeneration agent.

2.7. Purification of monoclonal antibodies from CHO cell culture supernatants

Thirty-five milligrams of the peptide resins, HWRGWV, HFRRHL or HYFKFD, were dry packed in 0.1 mL columns, swollen and washed as described in Section 2.2. After washing, the resins were equilibrated with PBS + 1 M NaCl. Samples of 100 μL of CHO cell culture supernatants were loaded onto the columns at flow rate of 0.05 mL/min (87 cm/h). The unbound proteins were washed from the column by using 4 mL of equilibration buffer. Product elution was carried out by using 4 mL of 0.2 M sodium acetate buffer, pH 4, or 0.2 M glycine–HCl, pH 3. Cleaning was performed by 4 mL of 0.85% (v/v) phosphoric acid and regeneration was done by 4 mL of 2 M urea in sodium acetate pH 4 buffer. All the chromatographic steps after loading were performed at a flow rate of 0.4 mL/min (693 cm/h). Chromatographic fractions were collected and analyzed for purity and yield using the methods described in Section 2.4. In the purification of MAb1 all three resins were tested (HWRGWV, HFRRHL and HYFKFD), while in the purification of MAb2 only the HWRGWV resin was used.

2.8. Determination of host cell protein (HCP) content

The host cell protein contents of the chromatographic fractions were determined using HCP ELISA kits from Cygnus Technologies (NC, USA). The high sensitivity protocol described by the manufacturer was used in the analysis. Briefly, the anti-CHO coated microtiter wells were filled with 100 μL of standards and samples and incubated on a rotator at 200 rpm for 1 hour at room temperature. The wells were then washed three times with 300 μL washing buffer and 100 μL of anti-CHO:HRP was added to each of the wells. These wells were incubated for 2 h under the same conditions as described previously. After incubation, the wells were washed four times and filled with 100 μL of 3,3′,5,5′ tetramethyl benzidine (TMB) substrate solution. The color was allowed to develop for 30 min at room temperature. The reaction was terminated by adding 100 μL of 0.5 M sulphuric acid to each well. The amount of hydrolyzed substrate was measured by μQuant Microplate reader (BioTek Inc., VT, USA) at 450 nm. A 4-parameter logistic fit was used to calculate the HCP (ng/mL) content in the chromatographic fractions. The HCP values determined in terms of ng/mL were converted into ng/mg of MAb to take into account the dilution of fractions. The log reduction value (LRV) obtained was determined by taking a $\log_{10}$ ratio of HCP in the load to the HCP in the elution fractions.

2.9. Determination of DNA content

The DNA content of the fractions was determined by quantitative polymerase chain reaction (qPCR) using a TaqMan® Residual CHO DNA Detection Kit (Applied Biosystems, CA, USA) and a 7500 Fast system RT-PCR (Applied Biosystems, CA, USA). All the chromatographic fractions were subjected to buffer exchange with 10 mM Tris–HCl, pH 7.5 using an Amicon® Ultra centrifugal filter (3000 MWCO, Ultracel®, Millipore, MA, USA). CHO DNA standards were prepared from the stock DNA solution (30 ng/μL). Ten microliters of standards, sample of different dilutions and controls, all in triplicates were added to the 96-well plate. Then, 20 μL of PCR reaction mixture consisting of the primers, probes and internal positive control were added to the wells. The plate was set up on the RT-PCR system and reaction carried out according to the manufacturer's protocol.

2.10. Determination of dynamic binding capacities

The three peptide resins HWRGWV, HFRRHL and HYFKFD were packed in chromatographic columns, washed and equilibrated with

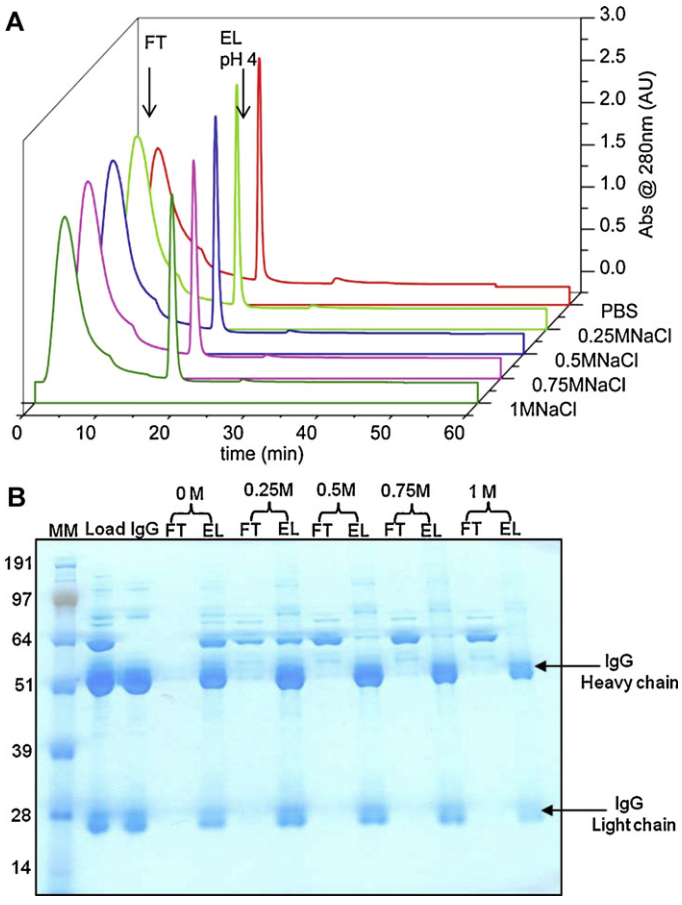

Fig. 1. (A) Chromatograms of purification of IgG from cMEM-IgG (10 mg/mL) mixture with HWRGWV resin under different NaCl conditions used during equilibration and binding. (B) SDS-PAGE (reducing conditions) of flowthrough and elution fractions. Labels: MM – molecular weight marker; FT – flowthrough fraction; EL – elution fraction.

PBS + 1 M NaCl as described in Section 2.7. An acetone pulse (5%, v/v) was applied to the columns to determine the total column void volume. Two milliliters of CHO cell culture supernatant containing humanized MAb1 was then loaded directly onto each of the peptide columns. Chromatographic fractions of 0.25 mL were collected and analyzed using a HiTrap Protein G column. Breakthrough volume was determined at the point where MAb1 concentration in the flowthrough fraction reached 10% of its feed concentration. This breakthrough volume was corrected by subtracting the void volume and based on this corrected volume the dynamic binding capacity of the peptide resins was determined.

3. Results

3.1. Influence of NaCl concentration on IgG yield and purity

Three cycles of purification of IgG from a cMEM mixture spiked with IgG (10 mg/mL) were carried out for each NaCl concentration (0 M, 0.25 M, 0.5 M, 0.75 M, 1 M) in the equilibration and binding buffers. The purifications were carried with the HWRGWV-Toyopearl resin. Fig. 1 shows the chromatograms and SDS-PAGE results of the runs with the different NaCl concentrations in the different equilibration and binding buffers. The purity of eluted IgG determined by densitometric analysis and the yield estimated by Protein G HPLC is shown in Fig. 2. It can be observed from the SDS-PAGE results (Fig. 1B) that, with increases in the NaCl concentration, the amount of the major impurity, albumin, in the flowthrough fraction increases while that in the elution fraction decreases. Fig. 2

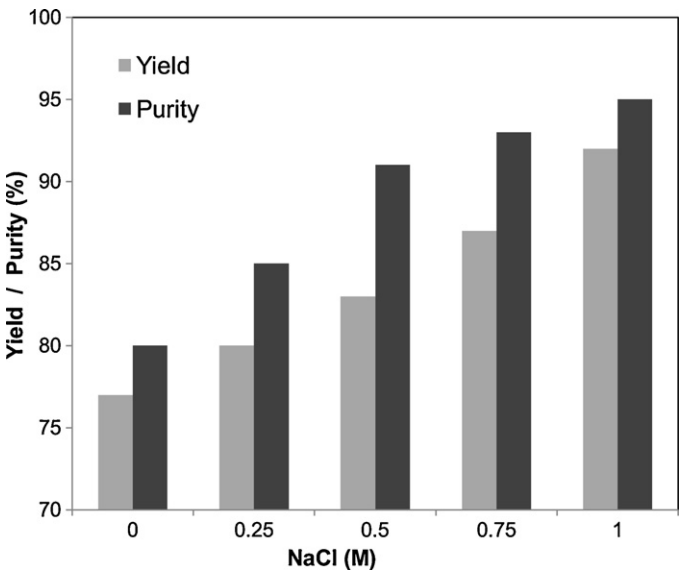

Fig. 2. Influence of NaCl concentration in the equilibration and binding buffer on yield and purity of IgG purified from cMEM-IgG mixture using HWRGWV resin. Data presented are averages of triplicate runs.

shows that both the yield and purity of IgG increased with increased NaCl concentration in the equilibration and binding buffers. The purity increased from 80% to 95% with increases in NaCl concentration from 0 M to 1 M. The yield of IgG increased from 77% to 92%. These results confirm that the non-specific binding of albumin is electrostatic, and is mostly due to presence of free amino groups of the base matrix or/and some truncated peptides that are formed during the solid phase synthesis of peptide on the polymethacrylate-based Toyopearl Amino resin. The presence of NaCl prevents the non-specific electrostatic binding of albumin to the amino groups in the resin, making the peptide groups freely available to IgG binding and thereby increasing the binding capacity.

3.2. Influence of sodium caprylate on IgG yield and purity

Albumin binds to several fatty acids such as caprylate to form a stable complex. BSA has at least ten binding sites for caprylate [34]. The high affinity interaction between caprylate and albumin could be exploited to reduce the nonspecific binding of BSA to the peptide resin. During purification of IgG using commercial affinity resins such as Mabsorbent A2P [35] and MEP Hypercel [36] it is recommended to have post-load wash steps with buffers containing low concentrations of sodium caprylate to remove the bound albumin. It was therefore decided to investigate the performance of HWRGWV resin under different concentrations of sodium caprylate (0 mM, 25 mM, 50 mM and 75 mM). PBS buffer containing 0.25 M NaCl was used as a base buffer since it was found that, in the complete absence of NaCl, there was no influence of sodium caprylate in improving the purity of IgG (data not shown). The chromatograms and SDS-PAGE analysis of the IgG purification from cMEM using different concentrations of sodium caprylate are shown in Fig. 3. From the SDS-PAGE analysis it can be seen that the addition of sodium caprylate increases the amount of BSA in the flowthrough, thereby increasing the purity of eluted IgG. Increasing the sodium caprylate concentration from 0 mM to 50 mM increased the IgG purity from 78% to 94%. Further increases in sodium caprylate concentration to 75 mM increased the purity of IgG to 97% but there was a significant loss of IgG in the flowthrough fraction, as can be observed in Fig. 3B, lane 75 mM FT. In this case the higher amount of sodium caprylate might be masking hydrophobic interactions of the HWRGWV resin

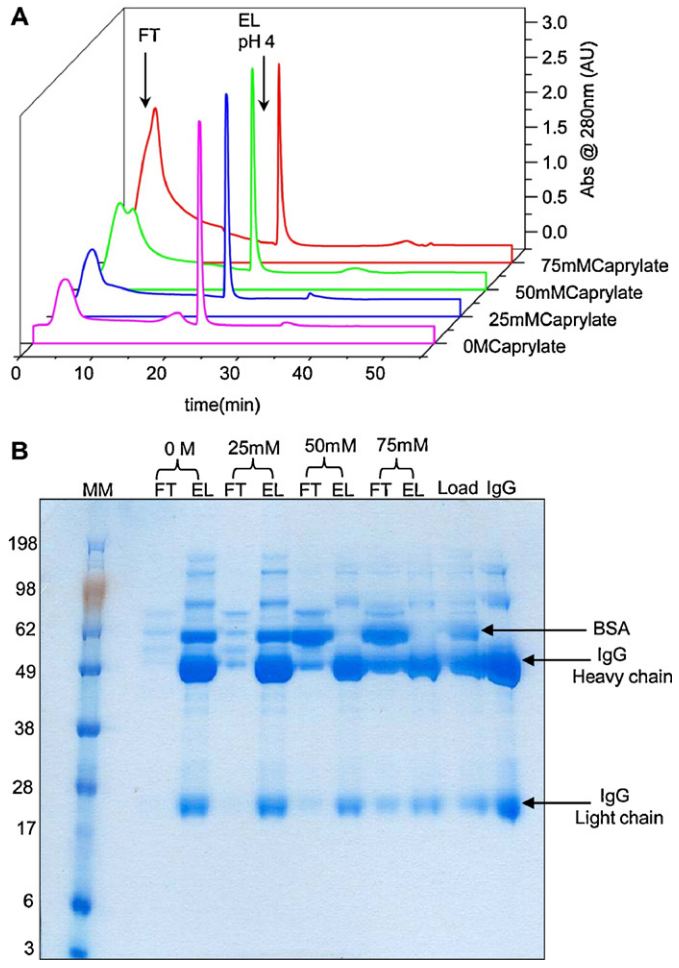

Fig. 3. (A) Chromatograms of purification of IgG from cMEM-IgG (10 mg/mL) mixture under different concentrations of sodium caprylate in the equilibration and binding buffer using HWRGWV resin. (B) SDS-PAGE (reducing conditions) of flowthrough and elution fractions. Labels: MM – molecular weight marker; FT – flowthrough fraction; EL – elution fraction.

with IgG, and since hydrophobic interactions form an important part of peptide-IgG binding [31], this leads to a decrease in the IgG binding capacity of the peptide resin. As a result, the optimum concentration of sodium caprylate to obtain high purity and yield of IgG is about 50 mM.

3.3. Batch screening of regeneration solutions

In earlier studies [30,31] column regeneration was performed with a 2% acetic acid wash. However, repeated use of the regenerated column (15 cycles) under these conditions showed a loss of binding capacity of HWRGWV resin (results not shown). As a result, batch studies were carried out to identify an efficient regeneration agent for HWRGWV-Toyopearl. Several commonly used regeneration agents were screened at different concentrations: sodium thiocyanate (1 M, 2 M, 3 M), urea (2 M, 5 M, 8 M), guanidine–HCl (1 M, 2 M, 6 M), 0.1 M glycine pH 2.5, 0.85% (v/v) phosphoric acid followed by 2 M urea in sodium acetate pH 4 buffer, 10% (v/v) ethanol, 10% (v/v) isopropanol and 50% (w/w) ethylene glycol. The regeneration efficiency results were calculated as a percentage (ratio) of the total protein desorbed relative to the total protein loaded onto the resins and are shown in Fig. 4. As can be observed, guanidine–HCl (2 M, 6 M) and 0.85% phosphoric acid followed by 2 M urea were the most effective regeneration agents in desorbing the proteins from the resins. In order to further confirm these results, the regen-

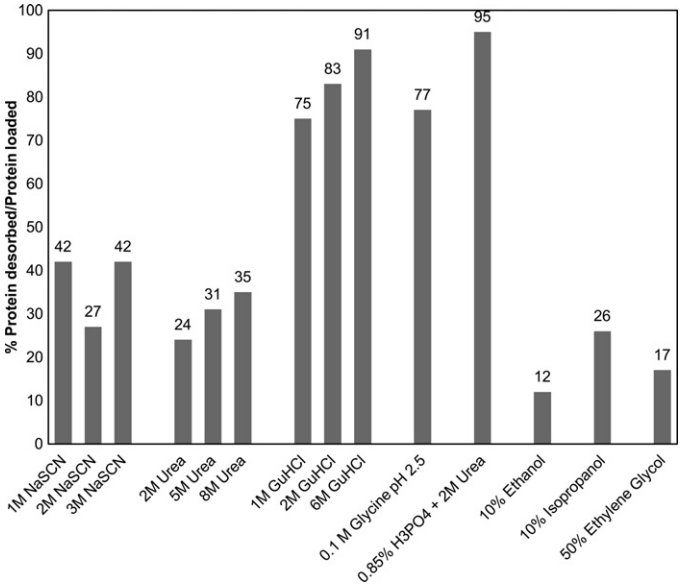

Fig. 4. Regeneration efficiency of HWRGWV resin (percentage of protein desorbed from resin to total protein loaded) using different regeneration agents.

erated resins were washed with PBS, boiled with the SDS buffer (mixture of NuPAGE® LDS buffer and NuPAGE® reducing agent) for 10 min and the supernatants were analyzed to determine the amount of protein still remaining bound to the resins. From Fig. 5 it can be seen that the resins regenerated with guanidine–HCl (2 M, 6 M) or 0.85% phosphoric acid followed by 2 M urea had the least amounts of bound protein, thus confirming their high regeneration efficiency. Since use of 6 M guanidine–HCl would lead to very high system pressure due to its high viscosity, only 2 M guanidine–HCl and 0.85% phosphoric acid followed by 2 M urea were selected for further chromatographic studies.

3.4. Secondary screening of the selected regeneration agents

Different cycles of IgG purification from a cMEM mixture using HWRGWV resin were carried out to more fully characterize the regeneration properties of the two most promising

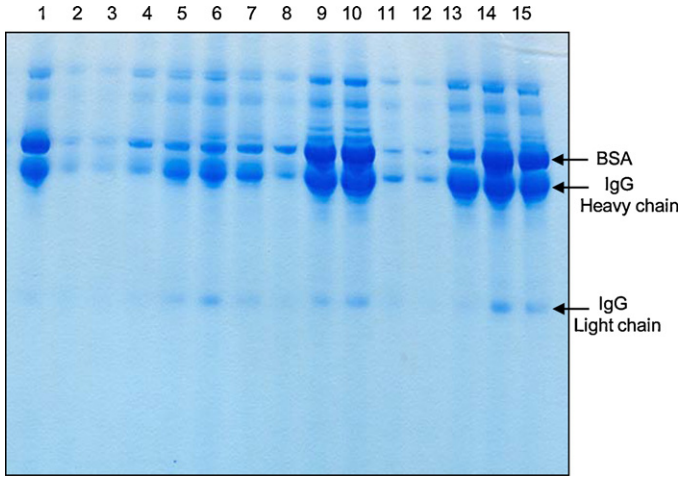

Fig. 5. SDS-PAGE (reducing conditions) of supernatants obtained by boiling the regenerated HWRGWV resin with SDS buffer. Lanes: (1) no regeneration agent, (2) 6 M GuHCl, (3) 2 M GuHCl, (4) 1 M GuHCl, (5) 5 M NaSCN, (6) 2 M NaSCN, (7) 1 M NaSCN, (8) 0.1 M glycine–HCl 2.5, (9) 2 M urea, (10) 5 M urea, (11) 0.85% phosphoric acid, (12) 0.85% phosphoric acid followed by 2 M urea, (13) 50% (w/w) ethylene glycol, (14) 10% (v/v) ethanol, (15) 10% (v/v) isopropanol.

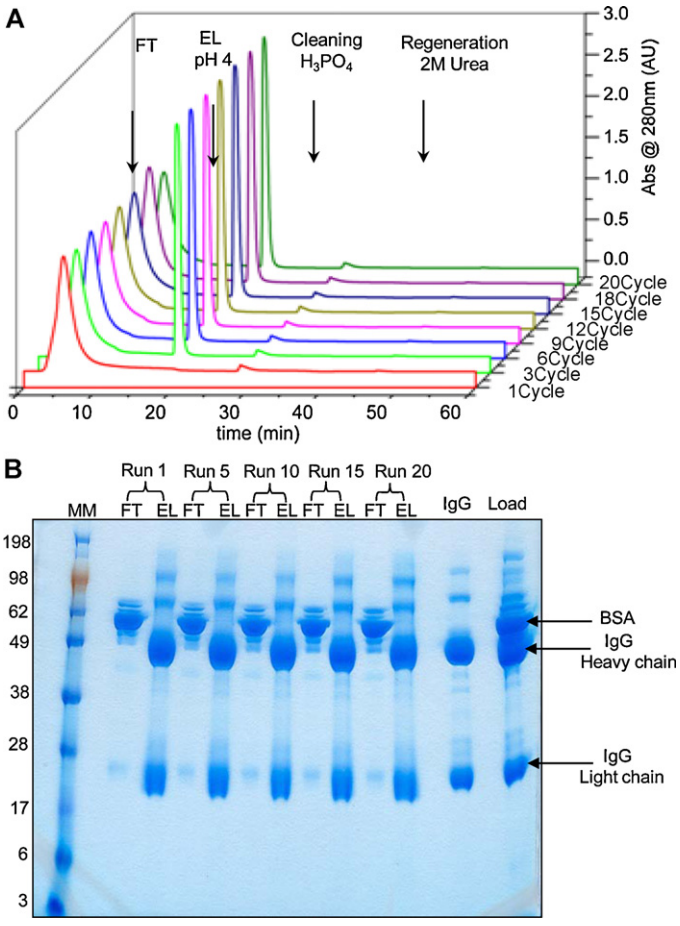

Fig. 6. (A) Chromatographic HWRGWV regeneration studies with 0.85% phosphoric acid followed by 2 M urea. (B) SDS-PAGE (reducing conditions) of chromatographic fractions. Labels: MM – molecular weight marker; FT – flowthrough fraction; EL – elution fraction.

buffer systems: 0.85% phosphoric acid followed by 2 M urea; and 2 M guanidine–HCl. The effects of reducing the concentration of guanidine–HCl from 2 M to 1 M were also studied.

3.4.1. Regeneration with 0.85% phosphoric acid followed by 2 M urea

Twenty cycles of purification of IgG from cMEM using HWRGWV resin were performed with a regeneration regime consisting of 0.85% phosphoric acid wash followed by 2 M urea. As can be seen from the chromatograms and SDS-PAGE results (Fig. 6) the performance of the peptide resin was consistent over this range of cycles. The IgG yield and purity over the purification cycles was 85% (±3%) and 94% (±2%), respectively.

3.4.2. Regeneration with 2 M guanidine–HCl

Twenty cycles of purification of IgG from cMEM using HWRGWV resin were performed with 2 M guanidine–HCl regeneration after each cycle. From the chromatograms (Fig. 7), it can be seen that the performance of the resin was maintained throughout the 20 purification cycles. The IgG yield for the purification cycles was 84% (±3%). For further purification cycles it was decided to test the use of 1 M guanidine–HCl instead of the 2 M concentration. As can be seen from Fig. 7, the performance of the peptide resin was consistent from the 20th cycle to the 30th cycle, indicating that even 1 M guanidine–HCl can efficiently regenerate the resin. Based on the above studies it can be concluded that both guanidine–HCl (2 M and 1 M), and 0.85% phosphoric acid followed by 2 M urea, were able to successfully regenerate the peptide resin.

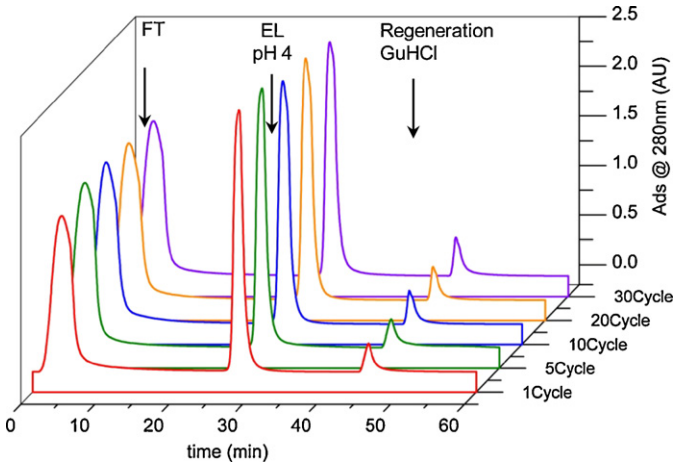

Fig. 7. Chromatographic regeneration studies of HWRGWV resin using 1 M and 2 M guanidine–HCl.

3.5. Purification of monoclonal antibodies from CHO cell culture supernatants

3.5.1. Purification of MAb1

HWRGWV resin was used for purification of a monoclonal antibody (MAb1) from a clarified cell culture fluid. MAb1 is a humanized MAb of IgG4 subclass and is a widely used therapeutic product. The concentration of MAb1 in the cell culture supernatant was 1.5 mg/mL. In addition to MAbs, the cell culture supernatant contained pluronic acid, yeast hydrolysate, cholesterol, tropolone, amino acids, glucose, vitamins and cellular components. The peptide resin was packed into the column and equilibrated with PBS + 1 M NaCl. The supernatant was then directly loaded onto the column without any pre-treatment. Washing, elution and regeneration steps were carried out as per the conditions optimized in the studies discussed in Sections 3.1 and 3.3. Washing was performed with 1 M NaCl and elution was performed with 0.2 M acetate buffer, pH 4. The chromatogram of purification of MAb1 is shown in Fig. 8A. It can be seen that the elution has a split peak. For comparison, a binding study of pure human polyclonal IgG at same concentration as that of MAb (1.5 mg/mL) was carried out under similar chromatographic conditions. In this case, a single peak was obtained during elution (Fig. 8B). The split peak in the case of MAb1 may be due to the presence of MAb aggregates in the cell culture supernatant that seem to bind more strongly to the ligand. Therefore, the purifica-

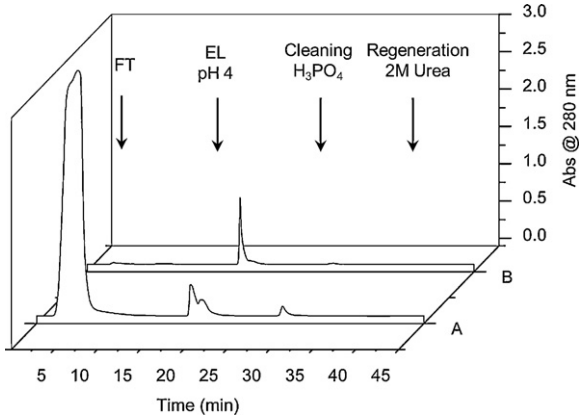

Fig. 8. (A) Chromatogram of purification of MAb1 (IgG4) from cell culture supernatant with elution at pH 4 using HWRGWV resin. (B) Chromatogram of binding of pure human polyclonal IgG under similar conditions as that used for MAb purification.

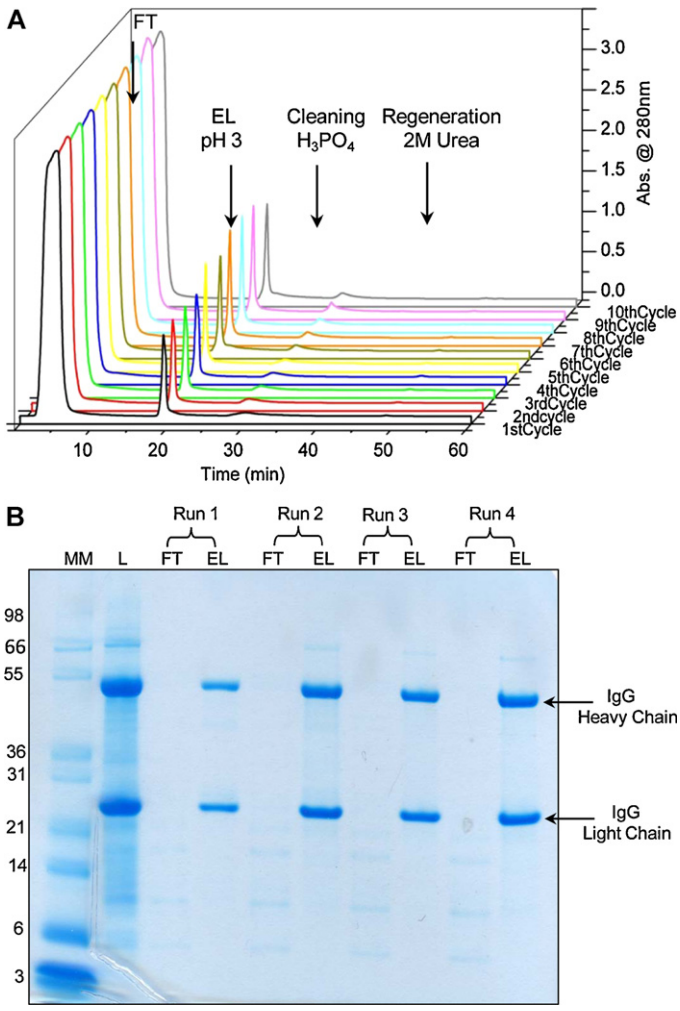

Fig. 9. (A) Comparison of ten cycles of purification of MAb1 (IgG4) from cell culture supernatant using HWRGWV resin. (B) SDS-PAGE (reducing conditions) of the chromatographic fractions. Labels: MM – molecular weight marker; FT – flowthrough fraction; EL –elution fraction.

tion of MAb1 was carried out at a lower elution pH of 3 using 0.2 M glycine–HCl buffer, resulting in a single elution peak (Fig. 9A). The SDS-PAGE of the chromatographic fractions (Fig. 9B) shows that the HWRGWV had selectively captured the MAb. The purity calculated by the densitometric analysis was 95% and the yield estimated by HPLC analysis was found to be 85%. As seen below, these results for yield and purity of the MAb1 compared very favourably with those obtained using a Protein G column.

The HCP and DNA content of the chromatographic fractions are shown in Table 1. The HWRGWV resin was able to reduce the HCP from 671 ng/mg MAb to 28 ng/mg MAb, achieving a log reduction value (LRV) of 1.4, which is comparable to the LRVs (1.4–2.3) reported for Protein A [37] and is better than those reported for mimetic ligands such as MEP Hypercel and MAbsorbent A2P [38]. The HWRGWV resin was also found to be very efficient in clearing the DNA, as no residual DNA was detected in the elution fractions.

Table 1
Host cell protein and DNA content of the chromatographic fractions of purification of MAb1 (IgG4) using HWRGWV resin.

Fractions	HCP (ng/mg MAb)	DNA (pg/mg MAb)
Load	671	1898.35
Flowthrough	90,052	68,337
Elution	28	ND

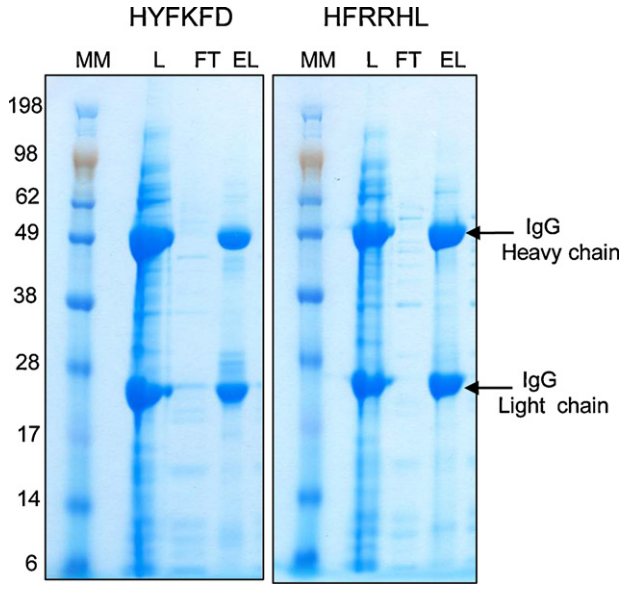

Fig. 10. SDS PAGE analysis (reducing conditions) of chromatographic purification of MAb1 (IgG4) using HYFKFD and HFRRHL resins. Labels: MM – molecular weight marker; FT – flowthrough fraction; EL – elution fraction.

To test the reusability of the HWRGWV resin, 10 cycles of MAb1 purification were carried out. Fig. 9A shows that the chromatograms of all the purification cycles were identical. The average purities and yields were 94% and 85% respectively, thus confirming the complete regeneration and reusability of HWRGWV resin within the range tested.

The two additional peptide resins, HYFKFD and HFRRHL, were also used for the purification of MAb1 from the cell culture supernatant. The conditions were the same as those used with HWRGWV resin. Five purification cycles were carried out with each of the two resins. The SDS-PAGE results of the MAb1 purification (5^{th} cycle) using HYFKFD and HFRRHL are shown in Fig. 10. From the SDS-PAGE it can be seen that both HYFKFD and HFRRHL selectively capture and purify MAb1. The average purity of all the runs was 93% and 95% for HYFKFD and HFRRHL respectively. The corresponding MAb1 yields were 86% and 84% respectively.

The performance of the peptide resins was then compared to results obtained using a Protein G column by carrying out purification of MAb1 using a HiTrap Protein G column. In order to keep the operating conditions similar to those used for the peptide resins, 1 mL of cell culture supernatant was loaded onto a 1-mL Protein G column (as 0.1 mL of supernatant was loaded on 0.1 mL peptide resin column). Equilibration was performed with 25 mM phosphate pH 7 buffer and elution was carried out with 0.1 M glycine–HCl pH 2.5 buffer (as per the manufacturer's instructions). The SDS-PAGE gels comparing the Protein G purification fractions and the HWRGWV elution fraction are shown in Fig. 11. It can be observed that the elution fraction of HWRGWV shows a purity similar as that of the Protein G elution fraction. Also, the MAb1 yield using the Protein G column was estimated to be 78%, which was lower than that obtained by HWRGWV (85%) and other two peptide resins. The reason for the low yields might be the presence of misfolded IgG, which might have the proper molecular weight but does not bind to either Protein G or to the peptide ligands due to blocked binding sites.

The performance of the peptide resins also compares well with other commercially available small ligands such as MAbsorbent A2P (ProMetic BioSciences, NJ, USA), MEP HyperCel (Pall Corp, NY, USA), and Kaptiv-GY (Interchim, Montlucon, France). MAbsorbent A2P, an aromatic triazine derivatized ligand was used to purify MAb

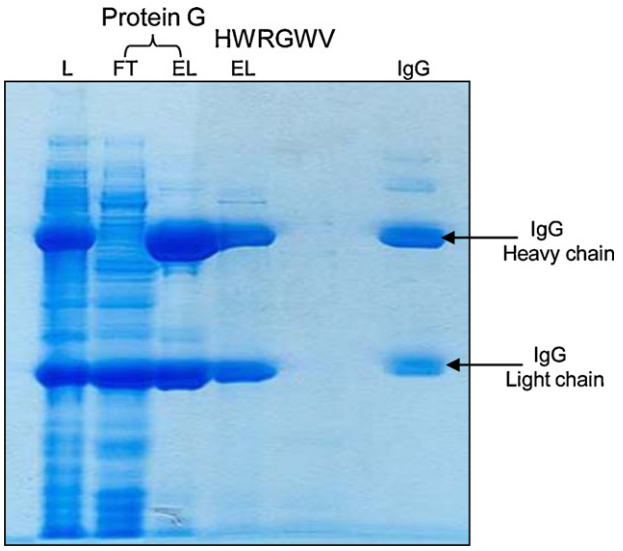

Fig. 11. SDS PAGE analysis (reducing conditions) of chromatographic purification of MAb1 (IgG4) using Protein G and HWRGWV resin.

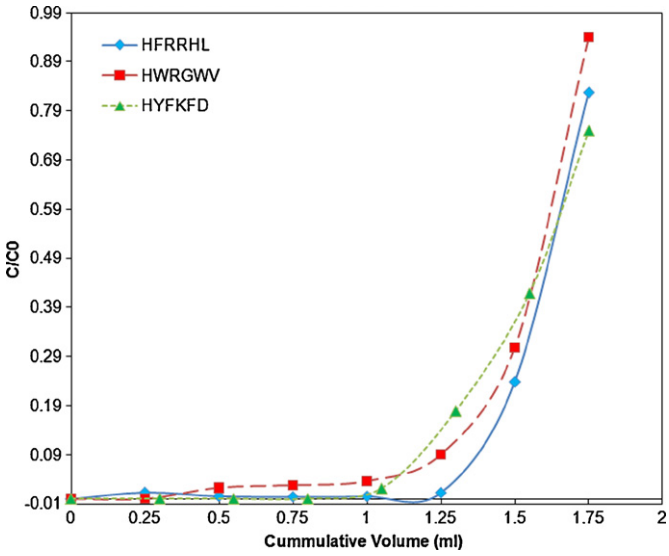

Fig. 12. Breakthrough curves of HWRGWV, HYFKFD and HFRRHL resins for MAb1 (IgG4).

from cell culture supernatant with both yields and purity above 95% [39]. However, its binding to IgG is weakened in the presence of pluronic acid, phenol red, surfactants and other hydrophobic moieties. Therefore, cation exchange chromatography was used prior to application of the antibody on MAbsorbent. Hydrophobic charge induction chromatography (HCIC) based MEP HyperCel resin has been used to isolate chimeric MAb from CHO cell culture supernatants resulting in yield and purity of 75% and 44% respectively [40]. In another application, MEP HyperCel was used for the purification of monoclonal IgG1 from cell culture supernatant containing 5% fetal bovine serum [13]. The MAb yield and purity in this case was 76% and 69% respectively. Kaptiv-GY, a tetrameric tripeptide was used to purify different classes of MAbs with yields and purity above 90% [22]. It is not known, with any of these ligands, whether the binding occurs specifically through the Fc fragment.

Table 2
Dynamic binding capacities of peptide resins for MAb1 (IgG4).

Peptide resin	DBC for MAb1 at 10% breakthrough (mg/mL of resin)
HWRGWV	18.4
HYFKFD	17.6
HFRRHL	19.3

3.5.1.1. Determination of dynamic binding capacity. The dynamic binding capacities (DBC) of three peptide resins for MAb1 were determined by breakthrough experiments. A 2-mL aliquot of CHO cell culture supernatant was loaded onto 0.1 mL of each of the three peptide resins at a flow rate of 0.05 mL/min (87 cm/h). The breakthrough curves are shown in Fig. 12. As shown in Table 2, the dynamic binding capacities of HWRGWV, HYFKFD and HFRRHL for MAb1 at the 10% breakthrough point were estimated to be 18.4 mg/mL of resin, 17.6 mg/mL of resin and 19.3 mg/mL of resin, respectively. Considering the fact that the breakthrough experiments were carried out in the presence of all cell culture components and impurities, the DBC obtained by the peptide resins is comparable to those generally reported for Protein A resins [41].

3.5.2. Purification of MAb2

The HWRGWV peptide resin was also used for purification of another monoclonal antibody (MAb2) from CHO cell culture

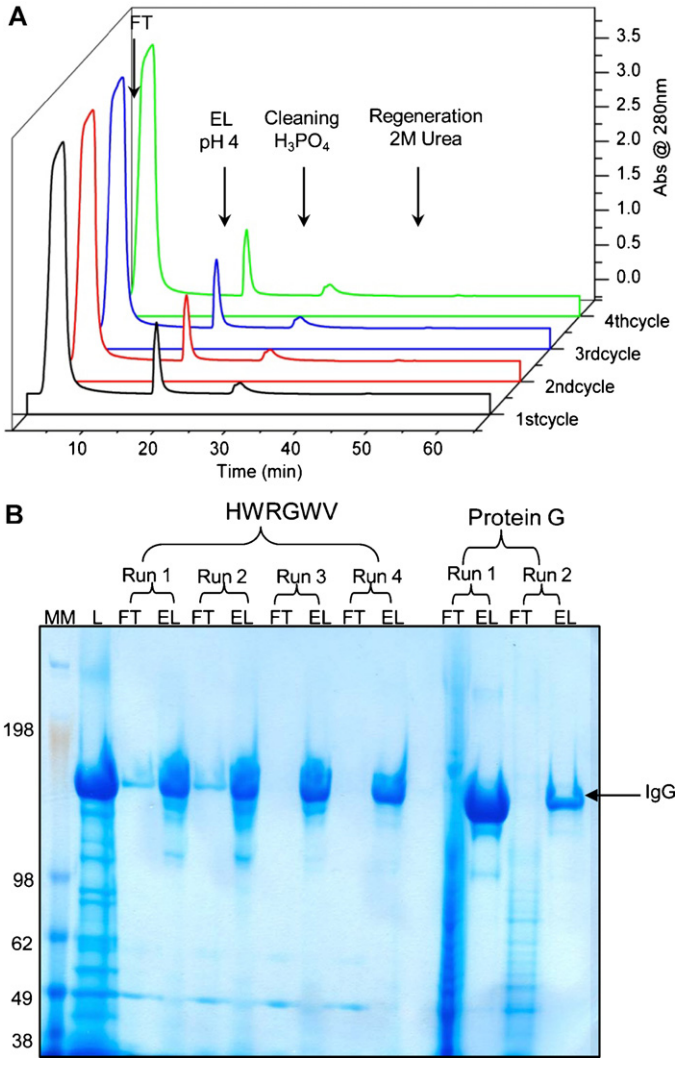

Fig. 13. (A) Chromatogram of four cycles of MAb2 (IgG1) purification from cell culture supernatant using HWRGWV resin. (B) SDS-PAGE analysis (non-reducing conditions) of the chromatographic fractions of MAb2 purification runs using HWRGWV and Protein G resins.

Table 3
Host cell protein and DNA content of the chromatographic fractions of purification of MAb2 (IgG1) using HWRGWV resin.

Fractions	HCP (ng/mg MAb)	DNA (ng/mg MAb)
Load	389,773	17,580
Flowthrough	15,128,536	278,993
Elution	9790	1.06

supernatant. MAb2 is a chimeric MAb of IgG1 subclass containing murine light and heavy chain variable region sequences and human constant region sequences. The concentration of MAb2 in the supernatant was 2.3 mg/mL. The peptide resin was packed into the column and equilibrated with PBS + 1 M NaCl. The cell culture supernatant was loaded directly onto the column without any pre-treatment. Elution was carried out using 0.2 M acetate buffer pH 4. The remaining chromatographic conditions were the same as used for purification of MAb1. Chromatograms and SDS-PAGE (non-reducing) analysis of four cycles of MAb2 purification using HWRGWV resin are shown in Fig. 13. The average yield and purity of the four purification runs was 84.5% and 95% respectively, which are similar to the values obtained for MAb1.

The host cell protein (HCP) and DNA content of the chromatographic fractions are shown in Table 3. The HCP levels were reduced from 389,773 ng/mg MAb to 9,790 ng/mg MAb resulting in a log reduction value (LRV) of 1.6, which is comparable to those reported for Protein A [37]. The peptide resin also showed a very high DNA clearance capability. The DNA concentration was reduced from 17,580 ng/mg MAb to 1.06 ng/mg MAb resulting in a LRV of 4.22, which is in the higher range of DNA LRV values (2.9–3.0) reported for Protein A matrices [42].

For comparison, a Protein G column from GE Healthcare (HiTrap Protein G) was used for the purification of MAb2 from CHO cell culture supernatant. A fraction of 1 mL of supernatant was loaded on a 1-mL Protein G column. All the operating conditions were same as used for the HWRGWV resin. The yield and purity (Fig. 13B) of MAb2 obtained were 82% and 96% respectively, similar to the values obtained with the HWRGWV resin.

4. Conclusions

The salt concentration in the binding buffer was found to be an important parameter during the purification of IgG by the hexamer peptide resin HWRGWV. Increasing the NaCl concentration in the binding buffer increased both the yield and purity of IgG. Using a 1 M NaCl concentration, purity and yields above 90% were obtained for the purification of IgG from cMEM. MS analysis (not shown) of the cleaved peptide from the base matrix reveals truncated peptide sequences formed during the solid phase synthesis of the peptide on the polymethacrylate based Toyopearl resin. These truncated sequences may be responsible for non-specific adsorption of albumin and other proteins. Since these truncated sequences have a terminal amino group, the interaction is primarily electrostatic at a pH 7.4, the pH used for the protein capture experiments. The addition of NaCl prevents the binding of these impurities and makes the peptide groups more freely available for IgG binding. In cases where the use of high NaCl concentrations is not convenient, it has been found that low concentrations of sodium caprylate (50 mM) can be used to achieve high IgG purity and yield. In this case, the sodium caprylate binds to specific sites on the albumin molecule to reduce nonspecific adsorption to the HWRGWV resin. If the sodium caprylate concentration is too high (75 mM or higher), it begins to interfere with the specific adsorption of IgG to the HWRGWV resin.

Efficient cleaning and regeneration agents are needed to ensure the consistent performance and long life of the resins. Various regeneration and cleaning agents have been reported in the literature and used commercially. For the HWRGWV resin, guanidine–HCl (1 M or 2 M) and 0.85% phosphoric acid followed by 2 M urea were found to be efficient washing and regeneration agents. Batch and column studies with cMEM confirmed the effectiveness of the selected regeneration protocols.

The three peptides HWRGWV, HYFKFD and HFRRHL were used to successfully purify a humanized MAb of IgG4 subclass. This MAb, which is a widely used therapeutic product, was purified from CHO cell culture supernatant with a purity and yield above 90% and 85% respectively. The yields and purity results obtained with the peptide resins were equal or better than results obtained with a Protein G column. The HCP and DNA reduction levels obtained with HWRGWV resin were comparable to values reported for Protein A resins. The dynamic binding capacities (DBC) of the three peptide resins for the MAb (IgG4) determined directly by loading the cell culture supernatant were in the range of 17–19 mg/mL of resin. The HWRGWV peptide resin was also used to successfully purify a chimeric MAb of IgG1 subclass from CHO cell culture supernatant. The purity obtained was 95% and the yield was 84.5%. The yield and purity obtained with the peptide resin were similar to those that obtained with a Protein G column. The HWRGWV resin was also very efficient in reducing the DNA and host cell proteins with log reduction values of 4.22 and 1.6 respectively.

Studies are in progress to characterize, validate and improve the solid phase synthesis of peptides on the resin to minimize truncated peptide sequences and obtain a highly homogeneous population of hexamer peptide on the resins. This should significantly enhance the binding capacity and specificity of the peptide resins thus resulting in a highly specific, small peptide affinity adsorbent for large-scale purification of antibodies and Fc fusion proteins.

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