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Purification of human immunoglobulin G via Fc-specific small peptide ligand affinity chromatography

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ABSTRACT

Chromatographic resins of a family of linear Fc-binding hexamer peptides (HWRGWV, HYFKFD, and HFRRHL) exhibited the ability to selectively adsorb and isolate human IgG (hIgG) from complete mammalian cell culture medium (cMEM). Among them, the HWRGWV resin with a peptide density of 0.08 mequiv./g of resin was able to purify hIgG from cMEM with both purity and yield as high as 95%, comparable to Protein A and A2P agarose gels. The influences of N-terminal acetylation of the HWRGWV resin, ligand density on the resin, initial hIgG concentration, and temperature on IgG isolation were also investigated. The results indicate that these small peptide ligands, especially HWRGWV, offer a potential alternative to the use of Protein A or Protein G for large scale affinity chromatography.

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1. Introduction

The increasing importance of antibodies as therapeutics [1] has led to a need for more efficient, robust, and lower cost processes to purify antibodies from serum, cell culture, and ascitic fluid. Chromatography plays a major role in industrial scale antibody purification. Ion-exchange chromatography (IEC) [2], hydrophobic interaction chromatography (HIC) [3], size-exclusion chromatography (SEC) [4], and affinity chromatography (AC) [5,6], have all been used in immunoglobulin G (IgG) capture and purification. Affinity chromatography allows the possibility of obtaining several fold purification with high recovery in fewer steps [7]. Half of all therapeutic antibodies currently in the market have been purified by affinity chromatography using Protein A or Protein G as ligands [8]. These ligands are costly because they have to be highly purified from bacterial sources; they have the potential for causing immunogenic response in patients upon possible leakage into the product, and they tend to lose activity as a result of harsh elution (pH 3), washing and sterilization conditions. As a result, there is a great deal of interest in identifying alternative affinity ligands for antibody purification.

Special attention has been paid to the use of small ligands for affinity purification of antibodies due to their advantages of being more stable (no three-dimensional structure), less immunogenic, and less expensive than large protein ligands. The use of high throughput screening techniques, sometimes combined with molecular modeling, has generated new and powerful small ligands for potential Protein A replacement. Roque et al. [9] have summarized several synthetic ligands, including peptidic and non-peptidic ligands, that have been discovered for antibody purification. Some of them have been extensively studied and are already commercialized, such as the hydrophobic charge induced ligand MEP (4-mercaptoethylpyridine) marketed as BioSepra MEP HyperCel [10–14], the Protein A mimetic peptide Kaptiv-GY based on the sequence $(RTY)_4K_2KG$ (TG19318) [15–17], the mixed-mode chromatographic ligand FastMabs A [18,19], and MAbSorbant A2P derived from a triazine derivative ligand 22/8 [20,21]. Meanwhile, the search for new ligands to be used in antibody purification is still quite intensive in both industry and academia [22,23]. Until recently [24], none of the small ligands developed to bind to antibodies behaved like Protein A in their binding specificity to the Fc fragment. The ability to bind through the Fc fragment is an important function since it allows the ligand to be used as a universal binder for whole antibodies and Fc-fusion proteins, potentially diminishing the time required to develop separation processes for new antibodies.

In a previous study [24], our group was able to show that the linear hexamer peptide ligands HYFKFD, HFRRHL, and HWRGWV, identified by a three-step screening process using a solid-phase hexamer peptide library, exhibited the ability to bind human IgG (hIgG) through its Fc portion. These peptide ligands share

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a common sequence homology consisting of histidine + aromatic patch + positively charged residue patch. The important Fc-binding property enables the use of these small ligands for binding Fc-fusion proteins, as well as orienting peptides on surfaces for immunoassays, therapeutics and diagnostics. In addition, HWRGWV was shown to be able to bind all subclasses of hIgG, hIgE, hIgD, hIgM, polyclonal IgGs from several other mammal and chicken species, and, to a less extent, secretory hIgA.

This article characterizes the ability of these linear Fc-binding peptide ligands to bind hIgG. Binding isotherms are presented with measured dissociation constants and equilibrium binding capacities. Their chromatographic performance in the purification of hIgG from complete mammalian cell culture medium (cMEM) containing 10% fetal calf serum (FCS) and 5% tryptose phosphate broth (TPB) were also determined. Among them, HWRGWV was studied in greater detail to determine the influence of temperature and ligand density on hIgG adsorption and separation.

2. Experimental

2.1. Materials

All peptide resins, HYFKFD, HWRGWV, HFRRHL, and N-terminal acetylated HWRGWV (Ac-HWRGWV) were synthesized directly on Toyopearl AF-Amino-650M (particle size 65 μm) (Tosoh Bioscience, Montgomeryville, PA, USA) using fluorenylmethyloxycarbonyl (Fmoc) chemistry by Peptides International (Louisville, KY, USA). All three peptide ligand resins were synthesized at a peptide density of 0.1 mequiv./g, while HWRGWV resins were also synthesized at different peptide densities ranging from 0.02 to 0.55 mequiv./g. Protein A Sepharose CL-4B resin (Protein A) was purchased from Amersham Biosciences (Piscataway, NJ, USA). MAbsorbent A2P immobilized on PuraBead 6% cross-linked agarose gel (A2P) was a gift from ProMetic Biosciences (Burtonsville, MD, USA). Phosphate-buffered saline (PBS) of pH 7.4, hIgG, sodium chloride, sodium acetate, monobasic sodium phosphate, dibasic sodium phosphate, glacial acetic acid were purchased from Sigma (St. Louis, MO, USA). Fab and $F(ab')_2$ fragments were from Calbiochem (Darmstadt, Germany). Fc fragments were from Bethyl Laboratories (Montgomery, TX, USA). NuPAGE Novex 4–12% Bis–Tris gels, NuPAGE MOPS running buffer, NuPAGE LDS sample buffer, NuPAGE reducing agent, Seeblue plus2 pre-stained molecular weight marker, and SimpleBlue SafeStain were all from Invitrogen (Carlsbad, CA, USA). hIgG enzyme-linked immunosorbent assay (ELISA) kit and Micro-BCA assay kit were from Alpha Diagnostic International (San Antonio, TX, USA) and Pierce (Rockford, IL, USA), respectively. Cell culture media EMEM was from Quality Biological (Gaithersburg, MD, USA). Fetal calf serum and tryptose phosphate broth were obtained from Hyclone (Logan, Utah) and Becton Dickinson (Sparks, MD, USA), respectively. MicroCon YM-3 filters (regenerated cellulose, 3000 MWCO) and Durapore 0.45 μm filters were purchased from Millipore (Billerica, MA, USA). Microbore columns of 30 mm long × 2.1 mm I.D. were from Alltech (Deerfield, IL, USA) and a Waters 626 LC system including a 2487 dual wave-length UV detector (Milford, MA, USA) was used for the chromatography separations. MGW Lauda RM6 circulating bath from Brinkmann (Westbury, NY, USA) was employed for temperature control.

2.2. Adsorption isotherm measurements

Adsorption isotherms of resins with different peptide ligands and different peptide densities were measured in a set of batch experiments at room temperature. All the experiments were performed at least in duplicate. Resins were weighed as dry powder, washed thoroughly and equilibrated with PBS at pH 7.4 containing 10 mM phosphate buffer, 2.0 mM KCl and 138 mM NaCl. Centrifugal filters (0.5 mL) with 0.45 μm Durapore membranes were used as adsorption vessels. Four hundred microliters (400 μL) of hIgG solutions with concentrations ranging from 0.05 to 10 mg/mL in PBS were added separately to the adsorption vessels containing 10 mg resin and incubated on an orbital shaker for 2 h. The unbound hIgG fractions were collected by centrifugation and the protein concentrations were determined using a Micro-BCA assay or by UV-absorbance at 280 nm. The amount of bound hIgG was calculated by mass balance. The data were fit to a Langmuir isotherm model

$$q = \frac{q_m C}{K_d + C} \qquad (1)$$

where q, C, K_d, and q_m are the concentration of the bound protein (mg-protein/g-resin), the concentration of the free protein (mg-protein/mL-solution), the dissociation constant (mg/mL), and the maximum capacity (mg-protein/g-resin), respectively.

2.3. Fragments of hIgG binding on HWRGWV

Fab, $F(ab')_2$, Fc fragments and intact hIgG binding on the HWRGWV resin were examined the same way as described in Yang et al. [24].

2.4. Chromatographic isolation of hIgG from cMEM

Resins were dry packed in 30 mm × 2.1 mm I.D. Microbore columns (0.1 mL) and washed with PBS. Before sample loading, columns were equilibrated with pH 7.4 loading buffer consisting of either PBS or PBS + 1 M NaCl. Human IgG was spiked into cMEM to form a complex starting material with the desired hIgG concentration, ranging from 0.5 to 10 mg/mL. The cMEM was formulated by combining EMEM with 10% FCS and 5% TPB. The salt concentration and pH of the starting material at this state were the same as PBS (7.4). Sodium chloride was added to the starting material when PBS + 1 M NaCl was the loading buffer. Human IgG spiked in cMEM was otherwise directly applied to the columns without any adjustment. Samples were injected at a flow rate of 50 μL/min for enough time to pass the same volume of the loading buffer as that of sample loop. The flow rate was then increased for the remainder of the run to 0.2 mL/min. When PBS was used as the binding buffer, the column was washed sequentially with 2 mL binding buffer, 4 mL washing solution, 4 mL elution buffer, and then 4 mL 2% acetic acid in water to clean the columns. When PBS + 1 M NaCl was used as the binding buffer, the column was washed sequentially with 1 mL binding buffer, 4 mL elution buffer, and then 4 mL 2% acetic acid. The washing solution was either PBS + 1 M NaCl or PBS + 1 M NaAc (sodium acetate) and elution buffer was 0.2 M phosphate buffer (PB) at pH 4 or 6 or 0.2 M acetate buffer (AB) at pH 4. For Protein A and A2P, both immobilized on agarose gel, the chromatographic conditions followed the instructions from each manufacturer. The effluent was monitored by absorbance at 280 nm. The temperature was controlled by using a water bath. Collected fractions were directly subjected to ELISA assays for concentrated by centrifugation at 4 °C, 11,247 × g for 90 min using a MicroCon YM-3 filter for electrophoresis analysis.

2.5. Sample analysis for yields and purities

The protein profiles of chromatography samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under conditions previously described by Yang et al. [24] and the purity of hIgG peaks was calculated by densitometry measurement using software ImageJ 1.32j (National Institutes of

Health, Bethesda, MD, USA). The yield of hIgG purification was calculated according to the hIgG concentrations determined by ELISA using a human IgG ELISA kit from Alpha Diagnostic International.

3. Results and discussion

3.1. Isotherms and isolation of hIgG by hexamer peptide ligands

3.1.1. Adsorption isotherms

Adsorption isotherms of hIgG using resins with ligands HYFKFD, HFRRHL, and HWRGWV at a ligand density of 0.1 mequiv./g on Toyopearl AF Amino 650M, as well as two commercially available IgG purification resins used as positive controls (Protein A agarose CL-4B and MAbsorbent A2P agarose gel) were determined (Fig. 1) with hIgG concentrations in the range of 0.05–10 mg/mL in PBS at room temperature. A2P is a "Protein A mimic" synthetic triazine ligand developed by ProMetic Biosciences. The isotherm adsorption data were directly fitted to a Langmuir model and the obtained dissociation constant (K_d) and maximum capacity (q_m) of each resin are listed in Table 1. Since the A2P resin was supplied in 20% ethanol the binding capacity for this material was reported in units of mg/mL of gel. The capacity of all other resins was measured by dry weight. In order to allow for a direct comparison, the capacity data were converted into mg/mL-drained-gel using estimated swelling ratios for the Toyopearl 650M material (4.7 mL/g) and for the Protein A resin (4 mL/g). Table 1 also lists the surface coverage of hIgG on the resins calculated based on q_m and known surface area of the beads. The surface area of dry Toyopearl Amino 650M resin was 30 m^2/g, while the equivalent areas of Protein A and A2P resins are unknown.

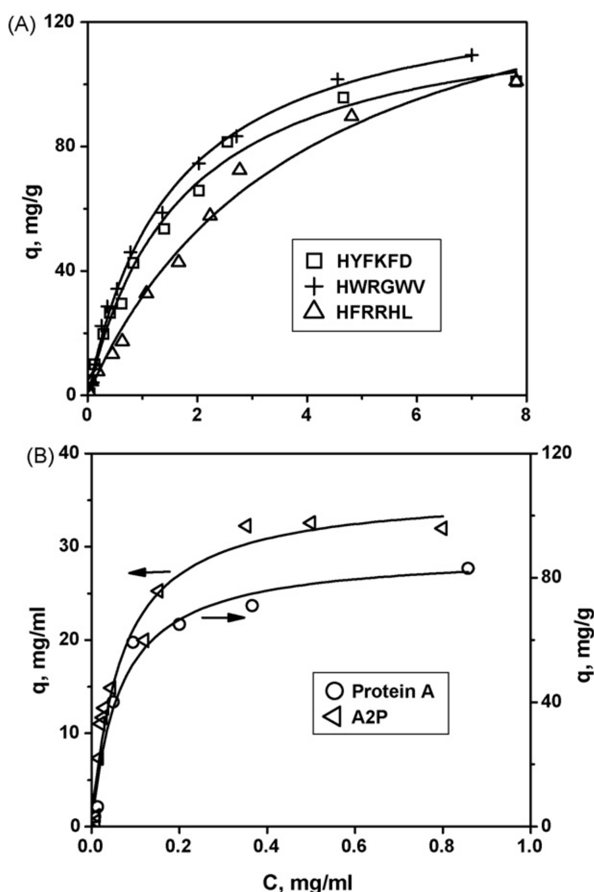

Fig. 1. Langmuir fits (lines) of isotherms for hIgG adsorption to resins of (A) hexamer ligands and (B) positive controls. The symbols are the original experimental data.

Table 1
Apparent dissociation constant K_d and maximum binding capacity q_m obtained using a direct fit of a Langmuir equation to the raw isotherm data as shown in Fig. 1. The ligand densities were 1.0 mequiv./g for all hexamer peptide resins.

Ligand	K_d ($\times 10^{-5}$ M)	q_m		R^2	C_s (mg/m^2)[a]
		mg/g	mg/mL[b]		
A2P	0.039	N/A	35.1	0.957	N/A
Protein A	0.043	88.1	22.0	0.966	N/A
HWRGWV	1.0	134	28.4	0.995	4.5
HYFKFD	1.1	127	27.0	0.991	4.2
HFRRHL	2.6	158	33.6	0.989	5.3

[a] C_s denotes surface concentration of hIgG which is calculated by dividing q_m by the surface area of the hexamer peptide resin beads (30 m^2/g).
[b] The swell ratios of Protein A and hexamer peptide resins are assumed to be 4 and 4.7 mL/g-dry-resin, respectively.

The maximum equilibrium binding capacities of the hexamer peptide resins at the ligand density of 0.1 mequiv./g were in the range of 27.0–33.6 mg/mL-drained-resin which was comparable to A2P resin, 35.1 mg/mL, and slightly higher than that of Protein A, 22.0 mg/mL. The capacities of the two positive controls derived from isotherms were in the range claimed by the manufacturers, 20–40 mg/mL for A2P and 20–30 mg/mL for Protein A. Based on the known hydrodynamic dimensions of IgG (235 Å × 44 Å × 44 Å) [25], closely packed "end-on" and "side-on" monolayers of unperturbed IgG molecules would give surface concentrations of 13 and 2.4 mg/m^2, respectively. Since the surface area of the base Toyopearl AF Amino 650M resin is approximately 30 m^2/g, a maximum binding capacity of 120–160 mg/g yields a surface protein density of 4–5.3 mg/m^2. This is of the same order of magnitude as the monolayer predictions, indicating that the hIgG molecules are forming a monolayer on the surface of the ligand resins when in equilibrium at saturation concentrations.

The dissociation constant (K_d) values for the peptide ligands were in the order of 10^{-5} M which are within the normal range (10^{-4}–10^{-8} M) for affinity chromatography resins [26]. This value also fell in the range of 10^{-5}–10^{-7} M usually found with short peptides as affinity ligand for proteins [27–29]. The K_d for the peptide resins were about two orders of magnitude larger than for the positive controls used in this study, as well as the reported values for ligands MEP Hypercel [12] and A2P [17]. However, as will be shown later, the weaker affinities can offer an advantage, since they allow for milder elution conditions of captured IgG. The milder elution condition may reduce aggregation and denaturation of the product. It is also possible for these ligands with dissociation constants of about 10^{-5} M to be used in quick and dynamic separation and to be operated in high-performance mode [30].

3.1.2. Peptide selection based on hIgG isolation from cMEM

In order to select a peptide resin for more detailed characterization, a preliminary purification experiment to test the ability of the peptide resins to isolate hIgG from cMEM was carried out. The yields and purities were not expected to be as high as possible in this experiment since buffer, peptide density, and other conditions had not been investigated. To simulate the purification of IgG from cell culture media, hIgG was spiked into cMEM, and the mixture was directly applied to 0.1-mL chromatographic columns packed with each of the three identified Fc-binding ligands (HWRGWV, HYFKFD, and HFRRHL). The peptide resins had a ligand density of 0.1 mequiv./g-dry-resin, except for HWRGWV, which had a slightly lower density of 0.08 mequiv./g-dry-resin. The columns were loaded in PBS, washed with PBS + 1 M NaCl to remove non-specifically bound proteins, and bound IgG was then eluted by 0.2 M phosphate buffer at pH 4. The purity and recovery of collected hIgG fractions (P2) were analyzed by SDS-PAGE and ELISA, and the results are listed in Table 2. HWRGWV, HFRRHL, and HYFKFD resins

Table 2
Recovery and purity of hIgG isolated from cMEM (duplicate runs) with different ligands in chromatographic formats. All data were obtained by using 0.1 mL columns with 1 mg hIgG loaded.

	Purity (%)	Recovery (%)
HWRGWV	90.9 ± 1.2	52.2 ± 1.0
HYFKFD	86.7 ± 3.2	42.0 ± 1.3
HFRRHL	88.6 ± 0.4	49.0 ± 0.4

demonstrated comparable results in terms of the ability to isolate hIgG from cMEM (Table 2). The preliminary chromatographic isolation of hIgG in one purification step achieved purities and recoveries of around 90% and 50%, respectively. They all were therefore good candidates for further investigation as antibody purification affinity ligands, with the possible less stringent elution conditions and proper capacities for hIgG. Since HWRGWV gave the highest purity and recovery, it was chosen for further study. The influence of peptide density, temperature, binding buffer, and elution conditions on hIgG isolation from the complex protein mixture are discussed in the following sections. It will be seen that both higher purity and recovery (≥95%) were obtained with HWRGWV, indicating its strong potential for antibody isolation from cell culture media.

3.2. Characterization of the ligand HWRGWV

3.2.1. Influence of peptide density on binding of hIgG fragments

The influence of peptide density on the binding of hIgG fragments was investigated, with the knowledge that HWRGWV at a ligand density of 0.08 mequiv./g bound hIgG specifically through its Fc region [24]. One hundred micrograms of pure hIgG and its fragments: Fc, Fab, and $F(ab')_2$, in PBS at pH 7.4 were separately injected into HWRGWV columns with different ligand substitution levels (0.022–0.55 mequiv./g). The retained target proteins were eluted with 2% AcOH. The percentages of bound proteins were calculated using peak areas and are shown in Fig. 2.

The peptide density on the HWRGWV resins had a significant influence on their binding of intact hIgG and hIgG fragments (Fig. 2). At a low density of 0.022 mequiv./g, the resin retained about 60% hIgG but had no efficient capture of any fragments. When the peptide density increased to 0.04 mequiv./g, more than 96% of Fc and hIgG were retained while less than 21% $F(ab')_2$ and even less Fab were bound. HWRGWV at 0.08 mequiv./g specifically bound the Fc fragment of hIgG, in similar amounts to the resin with peptide density of 0.04 mequiv./g. This indicates that a peptide density

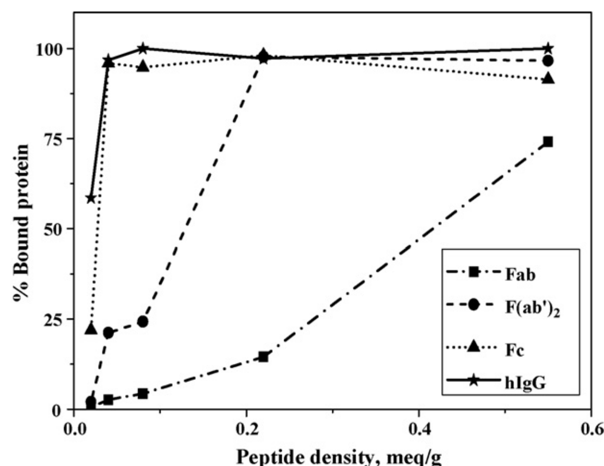

Fig. 2. Bound percentage of hIgG and its fragments to HWRGWV at different peptide densities. Fab, $F(ab')_2$ and Fc are fragments of hIgG. The percentage of a bound protein was calculated by the peak area.

Table 3
Apparent dissociation constant (K_d), maximum binding capacity (q_m), and superficial stoichiometry (S) at different ligand densities acquired by direct fit of the Langmuir isotherms to the raw data as shown in Fig. 3.

Density (mequiv./g)	q_m (mg/g)	K_d ($\times 10^{-5}$ M)	R^2	S (Peptide/IgG)
0.022	158.1	3.0	0.980	21
0.04	147.8	2.0	0.960	41
0.08	131.8	0.73	0.985	91
0.08, Ac-	130.8	0.38	0.972	92
0.10	133.6	1.0	0.995	112
0.22	136.8	0.26	0.963	241
0.55	137.6	0.095	0.911	600

threshold is necessary for HWRGWV to efficiently capture Fc and hIgG. With further increase of the peptide density to 0.22 and 0.55 mequiv./g, the resins retained the ability to bind Fc and intact hIgG but both bound higher percentages of Fab and $F(ab')_2$. The high peptide density apparently contributed to an increase in non-specific interactions with Fab and $F(ab')_2$ fragments.

With the knowledge that the isoelectric points of the majority of hIgG subclasses are in the range of 7–9, the majority of the IgG molecules are positively charged at pH 7.4, the same as the net charge on HWRGWV ligand at this pH. The similar charges on the ligand and the protein might suggest that electrostatic interactions are not the dominant driving force for binding of hIgG. Therefore, it could be expected that N-terminal acetylation should not influence the binding of hIgG or its fragments. This is supported by our experiments with Ac-HWRGWV ligand (data not shown) which exhibited no difference from HWRGWV on the binding of either intact hIgG or fragments of hIgG at the experimental pH value of 7.4, when amino groups are positively charged on the normal ligand. As for the other charged group in the ligand (the side amino group in arginine), it remains positively charged at the loading and elution pH values (7.4 and 4.0 or lower, respectively). As a result, its charge alone does not seem to have a marked influence on the capture of IgG.

3.2.2. Influence of peptide density on the adsorption isotherms of hIgG to HWRGWV

Resins with peptide densities of 0.022, 0.04, 0.08, 0.22, and 0.55 mequiv./g were prepared and their adsorption isotherms of hIgG in PBS were measured at room temperature (Fig. 3A). The adsorption data were fitted directly to a Langmuir model with the K_d and q_m values listed in Table 3. The average stoichiometry calculated as the ratio of ligand density to maximum capacity is also included in this table to indicate the number of peptides that might be in contact with one hIgG molecule at different ligand densities. The isotherm for Ac-HWRGWV at a peptide density of 0.08 mequiv./g is also reported in Fig. 3B and Table 3 for comparison. The correlation coefficients for the Langmuir fits to the isotherm data were very good ($R^2 > 0.96$) when the peptide densities were equal to or less than 0.22 mequiv./g but the fit was not as good ($R^2 = 0.91$) at the highest peptide substitution level (0.55 mequiv./g). This might be due to the non-specific adsorption of antibody through the Fab fragments at higher peptide density, in addition to the specific binding of Fc. This two-mode adsorption is not consistent with the uniform adsorption assumption of the Langmuir model.

The binding capacity decreased slightly at low peptide density with increases in the peptide substitution and then remained fairly constant after the peptide density reached values of 0.04 mequiv./g or higher, which was the threshold for efficient IgG capture. This observation is in contrast to the increased binding capacity with increasing ligand density observed with sorbents with less specific protein-ligand interactions, like hydrophobic chromatographic sorbents [31]. However, these results are similar to those reported by Wang and Carbonell [32] when the hexamer ligand YYWLHH

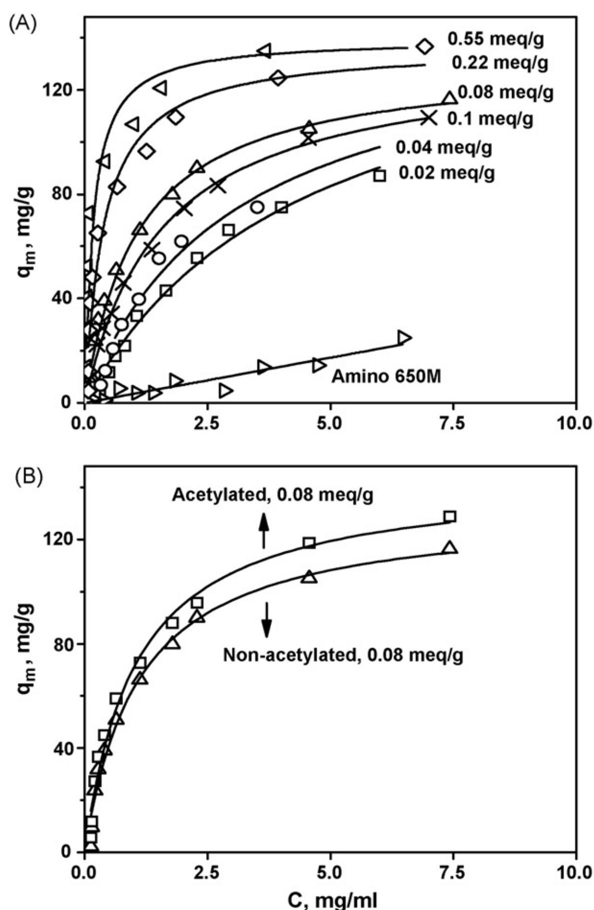

Fig. 3. Langmuir fits (lines) of isotherms for hIgG adsorption to (A) HWRGWV resins at different ligand densities and (B) to acetylated HWRGWV resin. Ligand densities are indicated in the graphs. Amino 650M is the base resin included as a negative control. Symbols are the experimental data.

was used to purify *Staphylococcal* enterotoxin B (SEB) spiked into *E. coli* lysate. Protein A has exhibited a similar behavior when tested for antibody and Fc-fusion protein capture [33], with the binding capacity being virtually the same when ligand density was changed (at the 50–100% substitution range where the density of a commercially available resin was set as 100%). The nearly constant capacity beyond the threshold density found in our study suggests that the surface might be totally covered by a monolayer of hIgG molecules regardless of the peptide density. Steric hindrance between IgG molecules upon binding to the surface leads to a marginal change in binding capacity with increasing ligand density.

The dissociation constants of hIgG adsorption decreased when the HWRGWV peptide density on the resin increased (Table 3). Decreased K_d values indicate a more intense, stronger, contact between the protein and the sorbent at higher ligand densities.

The stoichiometry of hIgG–ligand interaction progressively increased with the peptide density as indicated in Table 3. At low peptide densities, the ratio of ligand to protein at the maximum capacity was approximately 21:1, while at the highest peptide density the ratio was approximately 600:1. In view of the increased non-specific binding of Fab and $F(ab')_2$ at the higher peptide densities, the increased binding strength (decreased K_d) might be due to increased non-specific binding of hIgG Fab fragments.

The hIgG adsorption isotherm of Ac-HWRGWV resin was similar to that of HWRGWV at the same ligand substitution level, with the same q_m and K_d values within the experimental variance of $\pm 0.2 \times 10^{-5}$ M. This observation again suggests that the changes in ionic strength should not affect binding of hIgG since hIgG is not retained on the resin through electrostatic interactions.

As discussed above, HWRGWV with peptide densities of 0.04 and 0.08 mequiv./g specifically retained Fc fragments of hIgG, while higher affinity (lower K_d) was associated with higher density (0.08 mequiv./g). Therefore, a ligand density of 0.08 mequiv./g was used in most of our later studies to keep both the specificity and the affinity to the Fc portion of hIgG.

3.2.3. *Effects of peptide density and temperature on hIgG isolation from cMEM*

To study the effect of temperature and ligand density on the dynamic isolation of hIgG from cMEM solutions, chromatographic experiments were carried out using HWRGWV affinity resins at different peptide densities and at different temperatures. The preliminary chromatographic conditions as described in Section 3.1.2 were employed. Representative chromatograms and corresponding SDS-PAGE gels are shown in Fig. 4. The average purities and recoveries of eluted hIgG using pH 4 PB are listed in Table 4. When hIgG-spiked cMEM was applied to the HWRGWV column at 0.04 mequiv./g (Fig. 4A, dash–dotted line), three peaks appeared in the chromatogram: flow-through peak (FT), 1 M salt wash peak (P1), and pH 4 PB elution peak (P2). The first 3 lanes in Fig. 4B show the protein profiles of the three peaks, where P2 contained most of the hIgG, in its different isoforms (bands i–iv), as can be seen by comparing the respective bands in the standard hIgG lane (Fig. 4B, lane 8). The main contaminant in P2 was BSA in its monomer form at about 65 kDa. Most of the BSA present in the starting material seemed to have been held by electrostatic interactions in the HWRGWV column as it was eluted during the salt wash step (fraction P1, lane 2 in Fig. 4B). This made it possible to optimize the chromatographic conditions for purity and/or recovery improvement.

Fig. 4B also shows the SDS-PAGE gel of the three chromatographic peaks from HWRGWV resins with peptide densities of 0.04 (lanes 1–3) and 0.22 (lanes 4–6) mequiv./g and the hIgG peak P2 from densities of 0.08 (lane 10) and 0.55 mequiv./g (lane 11). P2 from an Ac-HWRGWV column under the same conditions is also included in Fig. 4B (lane 12). Only P2 is shown for the last two densities because the chromatograms of ligand density 0.08 and 0.55 mequiv./g had similar profiles to those of 0.04 and 0.22 mequiv./g, respectively. The band profiles obtained with 0.04 and 0.22 mequiv./g peptide densities were similar except for the different proportions of proteins in each fraction. More BSA was present in P2, while fewer proteins flowed through for the column with 0.22 mequiv./g HWRGWV resins. Both reduced FT and P1 fractions at higher ligand density could be due to increased non-specific interactions.

It is also noticeable by comparing the purities and yields achieved with different ligand densities at 21 °C (Table 4) that the four peptide densities could be sorted into two groups based on chromatographic performance: a low density (0.04 and 0.08 mequiv./g) group and a high density (0.22 and 0.55 mequiv./g) group. The purities and yields of the low density group were around 90% and 50%, respectively, while those of the high density group were approximately 80% for both the yield and the purity. Improved recovery at high density was compensated for the loss in purity of isolated hIgG, indicating that raising ligand density elevated not only the binding specific to hIgG but also the non-specific binding of competitive proteins. However, the disparity arising with ligand density was trivial unless the gap between the densities was significantly large.

Purities and yields of the runs at different temperatures are also tabulated in Table 4. The data show that increasing temperature did not affect the purity of the resulting hIgG but caused a slight decrease in the overall yield, similar to the findings for Protein A

Table 4
Purity (*P*) and recovery (*R*) of isolated hIgG from cMEM on the HWRGWV columns (0.1 mL in volume) at different ligand densities and temperatures. Densitometry and ELISA were employed respectively for *P* and *R* assessment.

Density								
	0.04 mequiv./g		0.08 mequiv./g		0.22 mequiv./g		0.55 mequiv./g	
T (°C)	*P* (%)	*R* (%)	*P* (%)	*R* (%)	*P* (%)	*R* (%)	*P* (%)	*R* (%)
4	/	/	91.1 ± 0.3	63.8 ± 3.8	/	/	85.7 ± 0.3	107.3 ± 0.9
11	/	/	89.6 ± 0.2	62.2 ± 2.3	/	/	84.6 ± 1.5	100.7 ± 1.3
21	91.7 ± 1.3	61.0 ± 0.2	90.9 ± 1.2	52.2 ± 1.0	84.2 ± 0.8	82.8 ± 1.9	83.9 ± 0.7	81.9 ± 1.6
38	/	/	88.5 ± 0.2	44.4 ± 0.1	/	/	81.8 ± 1.4	81.4 ± 7.4

that the differences of purities and yields between different temperatures were insignificant [34]. This suggests that the binding between hIgG and the ligand is not dominated by hydrophobic interactions. This also indicates that HWRGWV may be able to purify labile antibodies at low temperature without loss of its chromatographic performance.

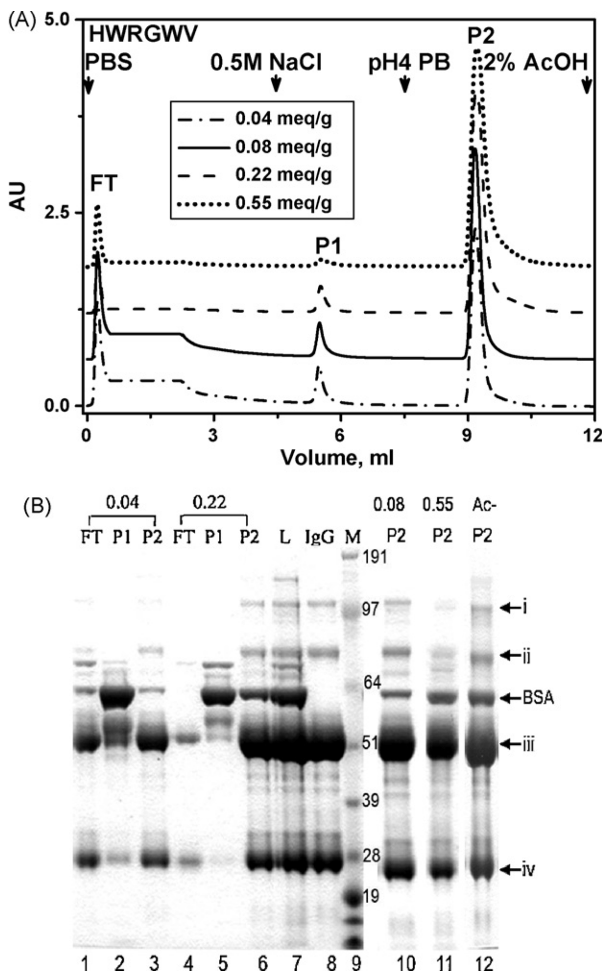

Fig. 4. (A) Chromatograms and (B) corresponding SDS-PAGE of hIgG separation from cMEM using HWRGWV resins at different densities. A: Columns were loaded in PBS at pH 7.4, and sequentially passed through 20 min each of 1 M NaCl for washing, pH 4 PB for elution and 2% acetic acid (AcOH) for cleaning. The flow rate was 0.2 mL/min, the concentration of hIgG 10 mg/mL and the injection volume 100 μL. The detected peaks were the flow through (FT), salt wash peak (P1), and PB wash peak (P2). The volumes of each collected peak were 2 mL. B: SDS-PAGE gel of the peaks denoted in panel A, as well as loading material (L) with a 1:5 dilution, standard hIgG (IgG), and molecular weight marker (M). The first two text lines mark the ligand density in mequiv./g and peaks of the chromatograms. Ac denotes another run on Ac-HWRGWV under the same chromatography conditions. Lables i–iv indicate the different combination of hIgG heavy (iii) and light (iv) chains. The gel was run under reducing conditions.

3.3. Enhancing yield and purity

This section describes the enhancement of the yield and purity by changing chromatographic conditions based on the observation that the isolated hIgG when loading in PBS was contaminated mainly by BSA. It was also evident that BSA was retained on the HWRGWV column predominantly by electrostatic interactions since the majority of BSA was eluted with 1 M salt. Two strategies were employed to improve the purification efficiency: (1) increasing the salt concentration by adding 1 M NaCl to the loading buffer, to eliminate BSA binding; and (2) lessening the elution stringency by using pH 6 PB to release only hIgG. Another buffer system, acetate buffer (AB), for washing and elution was also examined to determine if buffer ions affected the purification. All experiments were carried out at room temperature on a 0.1-mL column packed with 0.08 mequiv./g HWRGWV synthesized in a single batch by Peptides International. A 0.1-mL Ac-HWRGWV column (0.08 mequiv./g) was also investigated to better understand the nature of the ligand–protein interaction in the system. The yields and purities, analyzed respectively by ELISA and SDS-PAGE, of isolated hIgG are listed in Table 5 and compared to the results obtained with Protein A and A2P ligands. IgG was loaded in PB to the two commercial ligands as recommended by the manufacturers. Representative chromatograms and the corresponding SDS-PAGE gels are displayed in Fig. 5.

For the first strategy, high salt concentration in the binding buffer was employed to suppress BSA binding to HWRGWV, hence improving the product purity and yield (Table 5). To do this, the salt concentration in hIgG spiked cMEM was increased by adding NaCl to 1 M and the solution was then loaded to the column in PBS + 1 M NaCl at pH 7.4. Bound hIgG was released with 0.2 M PB at pH 4. Sodium chloride was chosen as the BSA binding–depressing salt because it has limited ability to promote hydrophobic interactions [35]. Increasing salt concentration of loading via addition of NaCl to 1 M effectively increased the chromatographic separation of hIgG on the HWRGWV column. The yield of isolated hIgG (Table 5) was boosted from 52% to 98% when the loading concentration of hIgG was 10 mg/mL.

Since both the hydrophobic and electrostatic interactions were excluded as the dominant forces for hIgG adsorption, the high salt concentration in the loading buffer seems to decrease the retention of BSA on the affinity medium instead of improving the hIgG binding. This is evident in Fig. 5A where a much larger flow-through peak was observed (Fig. 5A, HWRGWV) when compared to the FT fraction in PBS loading (Fig. 4); this flow-through peak contained most of the BSA (Fig. 5B, lane 1). The purity of isolated hIgG by HWRGWV ligand was also slightly increased at high salt loading (Table 5), even though a small amount of BSA monomers (62 kDa band) and dimers at ∼130 kDa (Fig. 5B, lane 2) were present. The presence of BSA monomers was similar to that of A2P (Fig. 5B, lane 16) and the binding of BSA dimers was probably due to non-specific hydrophobic interactions. BSA dimers, as well as the salt consumption, might be able to be reduced by using sodium caprylate in the loading buffer, since caprylate is known to interact with albumin and hence pre-

Table 5
Purity and recovery of hIgG product separated from cMEM at different chromatographic conditions. Data presented are the average of duplicate runs. Injection volume (V_0), initial hIgG concentration (C_0), loading buffer, elution buffer, and affinity media were varied in the investigation. The purity and yield were calculated by densitometry and ELISA, respectively.

V_0 (μL)	C_0 (mg/mL)	Elution buffer	Purity (%)	Recovery (%)
HWRGWV				
1 mg hIgG loaded in PBS				
100	10	pH 4 PB	90.9 ± 1.2	52.2 ± 1.0
100	10	pH 4 AB	91.0 ± 1.1	44.3 ± 1.6
100	10	pH 6 PB	93.0 ± 0.7	39.6 ± 0.3
1 mg hIgG loaded in PBS + 1 M NaCl				
100	10	pH 4 PB	94.8 ± 0.7	97.7 ± 1.0
100	10	pH 6 PB	97.7 ± 0.4	67.8 ± 0.8
Over-loading in PBS				
200	10	pH 4 PB	90.2	/
300	10	pH 4 PB	84.5	/
Ac-HWRGWV				
1 mg hIgG loaded in PBS				
100	10	pH 4 PB	81.4 ± 1.4	69.3
1 mg hIgG loaded in PBS + 1 M NaCl				
100	10	pH 4 PB	96 ± 0.6	95.5 ± 0.2
100	10	pH 6 PB	99 ± 0.3	69.7 ± 0.6
Over-loading in PBS				
300	10	pH 4 PB	81.7	/
300	10	pH 4 AB	80.3	/
Positive control, 1 mg hIgG loaded in corresponding concentration of PB				
Protein A				
100	10	pH 3 CA	100	96.9 ± 2.6
2 mL	0.5	pH 3 CA	98.7 ± 0.2	84.2 ± 4.9
A2P				
100	10	pH 3 CA	97.3	94.3 ± 1.0
2 mL	0.5	pH 3 CA	72.1 ± 4.5	52.7 ± 5.8

vent BSA from binding to columns [21,36]. When compared to the runs loaded in PBS, loading in high salt concentration progressively decreased the buffer volume for the chromatography process from 12 (Fig. 4A) to 4 mL (Fig. 5A) and decreased the process time by a factor of 3 at the same flow rate. Reduced processing time was another plus, along with the milder elution condition at pH 4, to keep the activity of antibodies. The high salt loading in PBS with 1 M NaCl was also applied on another two Fc-binding peptides HYFKFD and HFRRHL (Table 6). The high salt loading conditions resulted in higher yield and moderate improvement in purity. However, the increase in yield on HWRGWV was considerably larger.

The second strategy to increase yields and purity of hIgG was to elute the protein with PB at pH 6 instead of pH 4. The hope was that this would elute hIgG but not BSA from the HWRGWV column since BSA is predominantly retained by electrostatic forces. The isoelectric point (pI) of BSA is in the range from pH 4.6 [37] to 5.5 [38,39], depending on its isoform. It is also known that histidine becomes positively charged when pH decreases to below 6.0 while arginine and the N-terminal amine are always positively charged at acidic pH. When the elution pH was 4, positively charged BSA was extracted from positively charged resins together with hIgG. At pH 6.0 BSA is negatively charged and hence is retained by

Table 6
Initial values for purity and yield of IgG isolated from cMEM using resins HWRGWV, HYFKFD, and HFRRHL when the raw materials were loaded in PBS with 1 M NaCl.

Ligand	Yield (%)	Purity (%)
HYFKFD	65.8	92.0
HFRRHL	62.0	90.6
HWRGWV	94.8	97.7

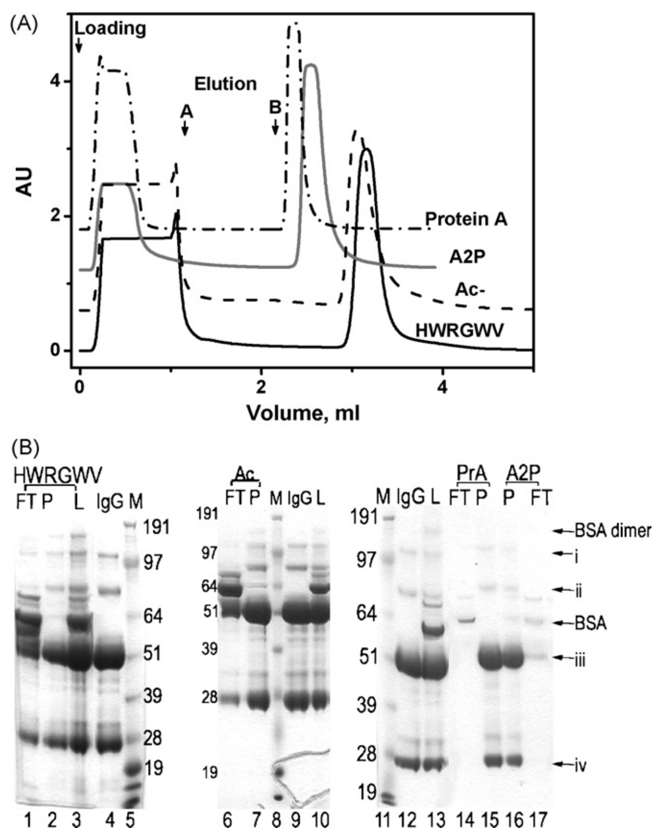

Fig. 5. (A) Representative chromatograms and (B) SDS-PAGE for hIgG separations from cMEM using different affinity resins. A: For HWRGWV and Ac-HWRGWV columns, protein mixtures were loaded in PBS + 1 M NaCl and bound hIgG was eluted with pH 4 PB. For Protein A and A2P, samples were handled according to manufacturer's specifications. Column volume: 0.1 mL; concentration of hIgG: 10 mg/mL; sample loop: 100 μL. B: Reduced SDS-PAGE gels of the chromatographic peaks on HWRGWV, Ac-HWRGWV (Ac-), Protein A (PrA) and A2P columns denoted in panel A, where the flow through (FT) and hIgG elution peak (P) for each column were analyzed, together with loading material (L) with a 1:5 dilution, standard hIgG (IgG), and molecular weight marker (M). Labels i–iv indicate the different combination of hIgG heavy (iii) and light (iv) chains. The gel was run under reducing conditions.

electrostatic interactions on positively charged HWRGWV resins. It is apparent from the data in Table 5 that, when using pH 6 PB to elute hIgG, the purity was increased from 91% to 93%. The purity increased from 95% to 98% by carrying out the adsorption step at high salt conditions. The purity of 98% achieved with pH 6 elution at high salt loading condition was comparable to those of Protein A and A2P. Bound hIgG being able to be eluted at pH 6, thanks to the weak interactions ($K_d = 10^{-5}$ M) between hIgG and HWRGWV, would be an advantage in isolation of pH sensitive antibodies. However, higher purity compensated the lower yield noting that only 75% of bound hIgG was eluted at pH 6.

Changing the salt type in the washing step from sodium chloride to sodium acetate and the elution buffer from phosphate buffer (PB) to acetate buffer (AB) had insignificant influence in separation (Table 5).

Experiments carried out by overloading the column with hIgG in PBS were performed on the HWRGWV column with injections of 20 and 30 mg/mL of hIgG instead of 10 mg/mL-gel, hoping that the components of stronger interactions (hIgG) could displace the components of weaker interactions (BSA), resulting in higher purity of product. The purities of isolated hIgG from the overloading runs are included in Table 5. The results in the table show that the displacement effect did not occur with the HWRGWV ligand, implying that an equilibrium state may be reached between the competitive

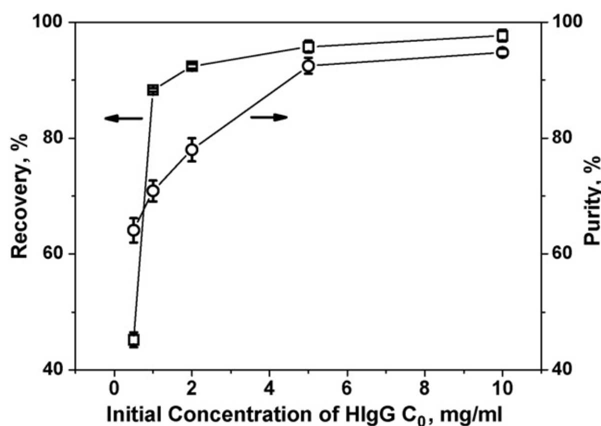

Fig. 6. Influence of initial hIgG concentration in cMEM on the purity and recovery of the product isolated by the HWRGWV column. Loading buffer: PBS + 1 M NaCl; elution buffer: pH 4 PB; column volume: 0.1 mL; amount of hIgG loaded: 1 mg.

binding of BSA and hIgG to the ligand under the previous loading condition of 10 mg/mL-gel.

Similar trends, qualitatively and quantitatively were observed when the above strategies were employed to purify hIgG from cMEM on an Ac-HWRGWV column (Table 5 and Fig. 5). Like HWRGWV, purity was improved from 81.4% to 96% when the salt concentration in the loading buffer was increased to 1 M and was further enhanced to 99% when pH 6 PB elution was coupled to the high salt loading condition. Meanwhile, the yield increased from 69.3% to 95.5% with the application of the high salt loading approach but dropped back to 69.7% when the two strategies were combined because of the partial elution. It can also be seen in Fig. 5 that when loading in high salt concentration, the protein profile in hIgG peak from Ac-HWRGWV resin (Fig. 5B, lane 7) was very similar to that of HWRGWV (Fig. 5B, lane 2). The similarity between the two ligands was also observed under PBS loading conditions (Fig. 4B, lanes 10 and 12), indicating that the N-terminal charges had negligible influence on capturing the main contaminant BSA retained by electrostatic interactions. Therefore, the charges on the side chain of arginine probably had a key role in BSA retention. It might be possible to block the charges on arginine to eliminate the BSA binding, but the influence of arginine on hIgG isolation is not known. Overall, it was demonstrated again that Ac-HWRGWV performed analogously to HWRGWV even in dynamic hIgG isolation, suggesting that the N-terminal charges played an insignificant role in both adsorption and isolation of hIgG from cMEM.

The initial concentration (C_0) of hIgG was found to influence the yield and purity of the isolated hIgG when the total loading amount was kept constant at 10 mg/mL-gel (C_0V_0 = 1 mg) (Fig. 6). Generally both purity and yield decreased with decreases in C_0 from 10 to 0.5 mg/mL. The purity, for example, diminished slightly from 95% to 93% when C_0 was reduced from 10 to 5 mg/mL, it fell from 93% to 78% when C_0 was reduced further from 5 to 2 mg/mL and kept dwindling with decreased hIgG concentration. The yield decreased slightly but remained higher than 88% when the hIgG initial concentration was decreased from 10 to 1 mg/mL and afterward the yield plunged from 88% to 45% when C_0 decreased from 1 to 0.5 mg/mL. When C_0 was reduced to 0.5 mg/mL, the purity and yield dropped, respectively to 64% and 45%, values that are much smaller than those obtained with Protein A but still comparable to the results obtained on the A2P column (Table 5) under similar conditions. It may be undesirable to apply the HWRGWV ligand for antibody purification from a source with antibody concentration of lower than 1 mg/mL. However, the ligand would be effective to be used for typical cell culture supernatants since a protein expression concentration titer of 1 mg/mL or higher is common in the industry [40].

The resulting purity and recovery on HWRGWV ligand resins obtained in this study were comparable to those from literature data of commercial small ligands: MAbSorbent A2P (Prometic BioSciences, Wayne, NJ, USA), MEP Hypercel (Pall Corp., East Hills, NY, USA), and Kaptiv-GY (Interchim, Montlucon, France). Resin A2P was optimized to purify polyclonal IgG from hyper immunized ovine serum with both the yield and purity greater than 95% [21]. HCIC has been studied for IgG purification, yielding purity and recovery of 44% and 75%, respectively, when the feedstock solution was from protein-free cell culture supernatant with IgG concentration at 0.0474 mg/mL [14]. When dealing with cell culture supernatant containing 5% FCS, the purity and recovery of hIgG were found to be 69% and 76%, respectively [13]. Kaptiv-GY isolated hIgG from human serum with both purity and yield of about 90% [22]. No literature data has been identified for this ligand in the purification of IgG from cell culture media.

Hexamer peptide HWRGWV exhibited the ability to purify hIgG from a medium containing 10% FCS and 5% TPB with purity and recovery, depending on the initial concentration of the antibody, comparable to the reported numbers listed above and the parallel experiments done on Protein A and A2P columns. However, HWRGWV was the only one among all of these small ligands known to mimic Protein A binding hIgG through its Fc portion. In addition, mild (up to pH 6) elution conditions could be applied to HWRGWV ligand to minimize the activity loss of the product, but with some loss in overall yields during the elution step.

4. Conclusions

Three Fc-binding linear hexamer peptide ligands (HWRGWV, HYFKFD, and HFRRHL) immobilized individually on Toyopearl AF Amino 650M resins showed dissociation constants in the range of 10^{-5}–10^{-6} M and the potential of isolating hIgG from cMEM. IgG was eluted at a less acidic condition (pH 4) than with Protein A and A2P ligands immobilized on cross-linked agarose gels. Among these hexamers, HWRGWV demonstrated the ability to purify hIgG from cMEM in one step with both the purity and the yield as high as 95% when hIgG concentration in cMEM was 10 mg/mL. These results are comparable to those obtained using Protein A and A2P resins. Lowering the initial hIgG concentration decreased the product purity yield, whereas it was still comparable to the results from the A2P resin when C_0 was 0.5 mg/mL. The larger than 88% recovery at hIgG concentrations higher than 1 mg/mL is promising for antibody capture from protein solutions like cMEM, namely serum containing cell culture supernatant.

The peptide density of HWRGWV had a significant influence on the binding affinity of hIgG and the specificity to the Fc fragment, but barely affected the binding capacity of hIgG. The peptide density also influenced the recovery and to a lower degree the purity of isolated hIgG. Resins with peptide densities of about 0.08 mequiv./g exhibited both the appropriate binding strength (7.3×10^{-6} M) to hIgG and the binding specificity to Fc fragments. Temperature had little effect on the chromatographic isolation of hIgG from cMEM when using HWRGWV as the affinity ligand.

N-terminal acetylation of HWRGWV had no influence on adsorption and isolation of hIgG from cMEM, indicating that the charge of the N-terminal histidine played no major role on the retention of hIgG to the HWRGWV ligand. Acetylation of N-termini also had negligible effects on capturing the main contaminant BSA, signifying that the charges on arginine probably were responsible for BSA retention. Studies on peptide column regeneration are currently being carried out, but preliminary data suggests the possibility to reuse the ligand for at least 35 cycles. The positive results shown

above indicate that small linear peptide ligands have great potential for use as Fc-specific ligands for antibody capture and purification.

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