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Abstract: Protein A mimetic peptide ligands have several benefits over conventional Protein A/G ligands, namely that they are small in size, have low production costs, are stable over a wide range of pH values and can withstand cleaning by harsh sanitization agents such as sodium hydroxide. In this paper, a hexamer peptide (HWRGWV) affinity matrix was used for the isolation of bovine immunoglobulins from various dairy streams (skim milk, acid whey and colostrum). Bound immunoglobulins were recovered in elution buffer (0.2 M sodium acetate buffer, pH 4.0) fractions with a purity of >85% in a single step. The peptide resin has achieved a maximum equilibrium adsorption capacity of 23±0.58 mg.mL-1 of resin for bovine IgG and had a dynamic binding capacity of 11.8±0.03 mg.mL-1 at residence time of 2 min. These results suggest that the hexamer peptide chromatography could potentially be used for the selective purification of bovine immunoglobulins from dairy streams. This method has promise as an alternative to conventional Protein A/G chromatography for direct capture of immunoglobulins from streams containing relatively high immunoglobulin concentrations such as colostrum, transgenic or hyper-immune milk.

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Dear Marcel Ottens,

Thank you for considering the above draft for the publication in Food and Bioproducts Processing Journal. I'm happy to address the missing information in revision1. I have now modified the manuscript with following changes.

Revision 2

Reviewer #1

Comment1.

In the abstract, equilibrium adsorption capacity should appear with the associated error (in the previous submission this error appear).

Decision: Authors are accepted the above and reviewed as suggested (23±0.58).

Comment2.

Page 12 line 10 "However, for experiments with standard bIgG at 1 mg mL-1 and 9 mg mL-1 were overloaded" The idea to answer comment 8, reviewer 1 is there but the sentence should better introduced in the text.

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Thank you.

Yours sincerely,

Jagan M Billakanti, PhD.

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Highlights

- HWRGWV peptide resin showed an equilibrium adsorption capacity of ~~21~~ 23±0.58 mg/mL of resin
- HWRGWV peptide resin showed interactions to both bovine IgG and IgA antibodies
- Bound antibodies were able to recover at mild elution conditions of pH 4.0
- Recovered antibodies showed >~~80~~85% purity for colostrum
- HWRGWV resin may be suitable for the purification of various antibodies from milk

Application of Peptide Chromatography for the Isolation of Antibodies from Bovine Skim Milk, Acid Whey and Colostrum

By

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Abstract

Protein A mimetic peptide ligands have several benefits over conventional Protein A/G ligands, namely that they are small in size, have low production costs, are stable over a wide range of pH values and can withstand cleaning by harsh sanitization agents such as sodium hydroxide. In this paper, a hexamer peptide (HWRGWV) affinity matrix was used for the isolation of bovine immunoglobulins from various dairy streams (skim milk, acid whey and colostrum). Bound immunoglobulins were recovered in elution buffer (0.2 M sodium acetate buffer, pH 4.0) fractions with a purity of >85% in a single step. The peptide resin has achieved a maximum equilibrium adsorption capacity of 23±0.581±0.58 mg.mL^{-1} of resin for bovine IgG and had a dynamic binding capacity of 11.8±0.03 mg.mL^{-1} at residence time of 2 min. These results suggest that the hexamer peptide chromatography could potentially be used for the selective purification of bovine immunoglobulins from dairy streams. This method has promise as an alternative to conventional Protein A/G chromatography for direct capture of immunoglobulins from streams containing relatively high immunoglobulin concentrations such as colostrum, transgenic or hyper-immune milk.

Keywords: Protein A mimetic, peptide ligands, affinity chromatography, bovine immunoglobulins, HWRGWV hexamer peptide, colostrum.

1 Introduction

The growing importance of bovine immunoglobulins as therapeutics and functional foods has provoked the need for innovative process technologies to isolate immunoglobulins (Igs) from dairy fluids. To date, most antibodies available on the market have been purified by affinity chromatography using either Protein A or Protein G ligands (Dancette et al., 1999; Farid, 2006; Fuglistaller, 1989; Hober et al., 2007). However, these ligands pose several inherent problems for process development because proteinaceous ligands are expensive and lose activity during the sanitization conditions generally applied during the column regeneration process. To overcome some of these drawbacks, a synthetic peptide ligand was used as an alternative affinity ligand and a chromatographic process for selective isolation of IgG from bovine milk fluids was developed.

Synthetic peptide based affinity chromatography processes have been successfully applied for the isolation of antibodies from human serum and cell culture media (D'Agostino et al., 2008; Fassina et al., 1996; Linhult et al., 2005; Menegatti et al., 2012). Small ligands such as peptides have potential advantages in chromatography processes because they can be more stable and less immunogenic than protein ligands, can be constructed with a wide variety of bio-specificity and their production cost is low (Fassina et al., 1996; Yang et al., 2005). Although several Protein-A Mimetic peptide ligands have been synthesized, screened and evaluated for their suitability in chromatography process development (Fassina, 2000; Roque et al., 2004; Yang et al., 2005), only a few have been extensively studied. For example, PAM peptide TG19318 (Fassina et al., 1998), a tetrameric peptide ligand, was successfully investigated for the isolation of various classes of human Igs (IgG, IgA, IgE, IgM) (Huse et al., 2002) from human serum and bacterial cell culture broths. Short linear hexameric peptide ligands HWRGWV, HYFKFD and HFRRHL

were identified and developed by Carbonell's group through screening of combinatorial libraries on a solid resin and its selectivity towards human Igs through the Fc region was characterised (Yang et al., 2005, 2009). Unlike protein A/G ligands, these hexameric peptides have a binding spectrum for all subclasses of human Igs (hIgG, IgD, IgE, IgM and, to a lesser extent, IgA) (Liu et al., 2012). They have binding interactions with IgG of different species such as bovine, rat, goat and mouse for which Protein A shows weak or no binding. Using the hexameric peptide ligands two commercial monoclonal antibodies were purified from CHO cell culture supernatants with good log reduction values for host cell proteins and residual DNA (Naik et al., 2011).

Systems such as milk and transgenic plants offer a potential economic alternative to CHO supernatants. However, the impurities encountered in these systems are different from those of CHO supernatants and therefore there is a need to study and develop purification strategies for these systems. Recently, a process comprising of a pretretament step and affinity chromatography using HWRGWV ligand was reported for purification of monoclonal antibody from transgenic *Lemna* plant extract (Naik et al., 2012). A few studies (Billakanti, 2009; Menegatti et al., 2012) have reported applications using hexameric peptide ligand for the isolation of IgG from dairy streams, which contain not only high levels of casein, whey proteins and lactose but also fat globules. Affinity chromatography processes using such challenging feed streams, where the value of the IgGs (polyclonal antibodies) is likely to be considerably lower than for recombinant therapeutic monoclonal antibodies, will require the advantages described above for small-molecule affinity ligands.

In this paper, therefore, we have investigated the application of the hexamer peptide, HWRGWV as an affinity ligand for the isolation of polyclonal bovine IgG (bIgG) from bovine milk streams.

2 Materials and methods

2.1 Reagents and instrumentation

The HWRGWV ligand was synthesized by Peptides International (Louisville, KY) on a Toyopearl AF-Amino-650 M solid-phase synthesis resin (particle size 65 µm) (Tosoh Bioscience, Inc., Montgomeryville, PA, USA) at a ligand density of 0.11 meq.g^{-1}. Individual bovine milk proteins, including α-lactalbumin (L5385), β-lactoglobulin (L3908), Bovine Immunoglobulin (bIgG, I5506), BSA (A7906) and caseins were purchased from Sigma-Aldrich New Zealand Ltd, Auckland, , New Zealand ~~(St Louis, MO)~~. Lactoferrin and lactoperoxidase were obtained from the Tatua Co-operative Dairy Company, Morrinsville, New Zealand. The AgResearch Group, Hamilton, New Zealand, kindly gifted bovine immunoglobulin A (bIgA). All other reagents, including sodium phosphate, sodium acetate and guanidine-HCl were of analytical grade and purchased from Sigma Aldrich New Zealand Ltd, Auckland, New Zealand.

For surface plasmon resonance (SPR) experiments, amine coupling reagents 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl (EDAC, 0.4 M), sulfo-*N*-hydroxysuccinimide (NHS, 0.1 M), ethanolamine-HCl (1.0 M, pH 8.5), sodium acetate buffer (10 mM, pH 5.0) and GLC biosensor chips were purchased from Bio-Rad Laboratories New Zealand Ltd, AUckland, New Zealand ~~(Hercules, CA)~~. Glycine-HCl buffer (10 mM, pH 1.5 and 1.75) and HBS-EP running buffer (containing 10 mM 4-2-hydroxyethyl piperazine-1-ethanesulfonic acid (HEPES), 150 mM NaCl, 3.4 mM ethylenediaminetetraacetic acid (EDTA), 0.005% P20 (Tween 20) surfactant at pH 7.4) were prepared in the laboratory using analytical grade chemicals purchased from Sigma Aldrich New Zealand Ltd, Auckland, New zealand. Affinity purified sheep anti-bovine IgG (A10-118, 1 mg.mL^{-1}) was purchased from Bethyl Laboratories (Montgomery, TX, USA). Skim

milk was purchased from a local retail store and whey was prepared from this as described below. Spray dried colostrum powder was purchased from a local health and nutrition store. A ProteOn XPR36 SPR system (Bio-Rad Laboratories New Zealand Ltd, Auckland, New Zealand) was used to determine the IgG concentrations as described by Billakanti et al. (2010). RP-HPLC was performed on an AKTA*explorer* 10 (GE Healthcare Life Sciences, Uppsala, Sweden).

For SDS-PAGE experiments, an Xcell4 *SureLock*™ Midi-Cell system (WRO100), NuPAGE Novex 4-12% Bis-Tris midi-gels (Lot No. 9081482, 1 mm x 20 wells), NuPAGE LDS sample buffer (4X) (Cat. No. NP0007), NuPAGE MES SDS running buffer (20X) (Cat. No. NP0002) and Novex Sharp pre-strained protein markers (P/N 57318) were purchased from Life Technologies New Zealand Ltd, Auckland, New Zealand.

2.2 Methods

2.2.1 Peptide chromatography column preparation

The hexamer peptide, supplied as a dry resin, was stored at 4°C for short term (<7 days) and was kept at -18°C for longer storage. Peptide resin (280 mg, dry weight) was swollen in 10 mL of 20% (v/v) methanol in water in a 15 mL falcon tube for 1 h in an end-to-end shaker. The swollen wet resin was then carefully packed without trapping air bubbles into an HR 5/10 column (5 mm i.d. x 10 cm length, GE Healthcare Technologies, Uppsala, Sweden) (total resin volume of 1 mL) and 20% aqueous methanol solution was passed through the column at a flow rate of 0.2 $mL.min^{-1}$ for 20 h. The peptide resin was then washed and equilibrated with 20 mM phosphate buffer saline (equilibration buffer) containing 1 M NaCl (20–30 column volumes (CV) prior to first use and 6 CV before all subsequent runs) at a flow rate of 1 $mL.min^{-1}$. Bovine IgG samples prepared in the equilibration buffer at pH 7.4 were injected onto the peptide column at a flow

rate of 1 mL.min^{-1}, followed by washing with equilibration buffer (5 CV) to remove unbound proteins. Bound proteins were eluted with 0.2 M sodium acetate buffer at pH 4.0 followed by column regeneration with 2 CV of a 2 M guanidine-HCl buffer.

Unless otherwise stated, the following buffer system was used for the experiments:

Buffer A (equilibration/binding buffer) contained 20 mM sodium phosphate and 1 M NaCl (84 mS.cm^{-1}) at pH 7.4; Buffer B (elution buffer) contained 0.2 M sodium acetate at pH 4.0; Buffer C (regeneration buffer) contained 2 M guanidine-HCl.

2.2.2 Standards and samples

Bovine IgG obtained from the commercial supplier was diluted in the equilibration buffer to approximately 10 mg.mL^{-1}. This stock solution was further diluted with equilibration buffer to give the desired concentrations and the solution absorbance was measured at 280 nm. The actual bIgG solution concentrations were calculated using the IgG extinction coefficient ($E^{0.1\%}_{1cm}$=1.26) (Farrell et al., 2004).

2.2.3 Milk, acid whey and colostrum sample preparation

Retail liquid consumer skimmed milk was purchased from a local store and acid whey was prepared from skimmed milk by heating to 45ºC in a water bath and adjusting the pH to 4.6 by drop wise addition of 5 M HCl. Precipitated caseins were removed by centrifugation at 18,500 x g for 20 min and the collected supernatant pH was then adjusted to 7.4 with 0.5 M NaOH. Finally, the acid whey solution was filtered through a 0.22 µm filter. The pH of skimmed milk samples was slowly adjusted to pH 7.4 by the drop wise addition of a 0.5 M NaOH solution. 1 g of colostrum powder was dissolved in 20 mL of the binding buffer and placed in an end-to-end

mixer for 60 min. The colostrum solution was then filtered through a 5 μm filter and the sample pH was adjusted to pH 7.4.

2.2.4 IgG estimation by SPR assay

An SPR biosensor assay was used for the determination of bIgG content in all liquid milk, colostrum and acid whey samples, following a protocol described previously by Billakanti et al. (2010) with minor modifications. Instead of using a blank channel for reference subtraction, this was performed using a double reference subtraction method (first with sample buffer and the second with inter-spot reference subtractions). In this way, use of an extra channel to remove buffer shift during analyte responses could be avoided. Six samples were run in parallel at 100 $\mu L.min^{-1}$ for 5 minutes and the SPR responses compared to standard curve and calculated the amount of bIgG in each sample while keeping the auto sampler temperature at 4°C and chip at 25°C. The amount of bIgG bound to the hexamer adsorbent was calculated from mass balances on the individual steps of chromatography experiments i.e. flow through, wash, elution and regeneration fractions. All experiments were performed in triplicate and average values were used in calculations and data representation.

2.2.5 IgG estimation by RP-HPLC

To evaluate the specificity of the hexamer peptide ligand towards IgG and its cross-reactivity with milk and whey proteins, it was necessary to examine all flow through, elution and regeneration fractions for each chromatography experiment. Reversed phase high-performance liquid chromatography (RP-HPLC) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analytical methods were used to evaluate the purity of IgG in the

elution fractions and cross-reactivity of the ligand with other components. The RP-HPLC method has been widely used in whey protein quantification (Elgar et al., 2000) but is not a suitable method for the quantification of whey proteins from skim milk and colostrum samples because of the occurrence of overlapping peaks between caseins and whey proteins. However, it could be used for qualitative estimation of purity in elution and regeneration fractions for each experiment on skim milk and colostrum samples. In the case of acid whey experiments, the RP-HPLC assay alone was used for both quantitative and qualitative analyses of IgG and whey proteins. For skim milk and colostrum loading experiments, both SPR (quantitative) and RP-HPLC (qualitative) methods were applied. The experimental protocol for RP-HPLC followed that previously described by Elgar et al. (Elgar et al., 2000). The qualitative purity (visible) of the selected elution fractions were further assessed by SDS-PAGE.

2.2.6 SDS-PAGE assay

SDS-PAGE analysis was performed following the supplier's protocol with minor modifications (Invitrogen, Auckland, New Zealand). Samples were prepared at appropriate concentrations using a ready-to-use sample buffer (5 μL of sample buffer + 15 μL of sample) and heated for 7 min at 90°C. Samples were then loaded into a 20-well Bis-Tris (4–12%) gel and the gel was run at a constant voltage of 200 V for 32 min. Gels were stained for 30 min in Coomassie Brilliant Blue 250 stain solution (0.25% (w/v)) and de-stained overnight. Finally, the gel image was scanned using the Bio-Rad Gel Doc system (Bio-Rad Laboratories).

2.2.7 Adsorption isotherm measurement

Batch experiments were performed using commercial bIgG at room temperature to determine the equilibrium adsorption capacity of the HWRGWV resin at various IgG concentrations. Aliquots

of 10 mg of the peptide resin (dry resin) were accurately weighed into 1.5 mL microcentrifuge tubes and swelled in 20% aqueous methanol overnight. The tubes were centrifuged (4000 x g, 5 min) and the supernatant carefully removed with a pipette to avoid loss of resin and the swollen resin was then equilibrated with binding buffer for 2 h. Commercial bIgG samples of known purity were reconstituted in the binding buffer at concentrations of 1–9 $mg.mL^{-1}$ and 300 µL bIgG solution of each concentration was added to each 10 mg resin tube and continuously mixed by gentle inversion on an end-to-end rotating plate for 24 h. The supernatant was then collected and the equilibrium bIgG (C^*_{IgG}) concentrations measured UV at 280 nm. Total bIgG adsorption on the peptide resin was then calculated from the difference between the initial and final concentrations of the bIgG in the solution phase. The average values of triplicates and standard deviations were used to generate isotherm fits.

To determine the dynamic binding capacity of the peptide resin at varying bIgG load concentrations, a series of 1 mL samples of bIgG(1–9 $mg.mL^{-1}$) were applied to 1 mL peptide column in separate runs at a constant flow rate of 1 $mL.min^{-1}$ (2 min residence time) . The dynamic binding capacity of peptide resin for each concentration was then calculated from a mass balance on the flow through and elution peaks

3 Results and Discussion

3.1 Adsorption capacity

The equilibrium adsorption isotherm obtained for commercial bIgG on ~~the hexamer~~the hexamer peptide resin is shown in Fig. 1 and the equilibrium adsorption capacity was described well by the Langmuir isotherm (equation 1). Values for the constants in this equation were calculated from the adsorption capacities measured at various concentrations of bIgG solutions.

$$Q = \frac{Q_o C}{K + C} \quad (1)$$

where Q is the adsorption capacity (mg bIgG adsorbed per mL resin) at equilibrium with a solute at solution concentration C (mg.mL^{-1}), and Q_0 (22.99 mg.mL^{-1}) and K (0.33 mg.mL^{-1}) are constants determined from the adsorption experiment. The peptide resin had a maximum equilibrium adsorption capacity of 21±0.58 3±0.58 mg.mL^{-1} of resin at C = 9 mg.mL^{-1}, which is similar to the equilibrium adsorption capacity (22 mg of human IgG per mL of resin)resin) of a commercial Protein A Sepharose™ CL-4B resin.

The dynamic binding capacity at a flow rate of 1 mL.min^{-1} (2 min residence time) was approximately 11.8±0.03 mg.mL^{-1} of resin at a feed concentration of 9 mg.mL^{-1}. As expected, the dynamic binding capacity of the resin was greatly dependent on the amount of bIgG present in the feed solution. For example, at 1 mg.mL^{-1} bIgG solution, total amount of IgG bound to the column wasthe dynamic binding capacity was 4.8±0.21 20 mg.mL^{-1}, which was approximately 40% of that of total IgG bound atat 9 mg.mL^{-1} of bIgG in the feed samples (Table 1.1)

The peptide ligand interactions with commercial bIgG and all other milk and whey proteins were investigated at prescribed buffer conditions (Section 2.2.1). During this study, various volumes (1–6 mL) and concentrations (1–9 mg.mL^{-1}) of pure bIgG samples were injected through the 1 mL peptide resin column. Most IgGs (>90%) bound to the column with a minor leakage (<10%) in flow through when applied required sample volumes. Bound IgGs were successfully recovered (>90%) during the elution with 0.2 M acetate buffer (buffer B, pH 4.0) (Table 1.1) and the remaining strongly bound IgGs were removed during column regeneration with 2 M guanidine-HCl. Application of commercial bIgG through the hexamer column confirmed that the peptide ligands interact with bovine IgG with only minor leakage (<10%) when applied required

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sample volumes to reach the breakthrough (Table 1.1). However, for experiments with standard bIgG at 1 mg.mL^{-1} and 9 mg.mL^{-1} were overloaded since column reached its dynamic capacity approximately at 5.5 mL for 1 mg.mL^{-1} bIgG and 1.4 mL for 9 mg.mL^{-1} bIgG. Due to this, % of IgG bound data presented in table 1.1 for these experiments may not reflect the actual numbers.

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Similarly, commercial individual milk and whey proteins (1 mL of 3 mg.mL^{-1}) were injected into the peptide column at a flow rate of 1 mL.min^{-1}. Cross-reactivity of each individual protein was estimated from the mass balance calculations (initial, flow through, elution and column regeneration). Peptide ligand showed a partial cross-reactivity for caseins and beta-lactoglobulin (β-lac) proteins at the given concentrations. All bound caseins were removed during column regeneration but β-lac was eluted at the same conditions as bIgG (Fig. 5).

The impact of buffer conductivity on bIgG binding to the peptide resin was further investigated using different amounts of salt (8.3–160 mS.cm^{-1}, 0.05–2 M of NaCl) in the binding buffer at pH 7.4. The binding capacity of peptide resin for bIgG increased with increase in buffer conductivity. For buffer conductivities >44 mS.cm^{-1} (0.5 M NaCl) the peptide resin showed 10–20% higher bIgG binding capacities over low salt binding buffers in the feed acid whey sample (Table1.1). On the other hand, buffer conductivity <44 mS.cm^{-1} resulted in differing levels of cross-reactivity with various milk and whey proteins (β-lac, α-lac, LF and caseins (mainly β-caseins), Table 1.2).

The amount of cross-reactivity of peptide resin towards milk proteins was mainly dependent on binding buffer conductivity and the amount of bIgG present in feed solutions. For example, all of whey proteins were bound (only a small UV trace observed in flow through) to the peptide column when 1 mL of whey sample loaded at a conductivity of 8.3 mS.cm^{-1} and pH 7.4 but increase of whey loading (>3 mL whey) resulted in replacement of β-lac by α-lac, LF, caseins

and bIgG (data not shown). These results indicate that the peptide ligand weakly adsorbs bIgG at low buffer conductivities and has high cross-reactivity with β-lac.

Cross-reactivity of peptide resin was further investigated using frontal analysis of acid whey loading through the 1 mL peptide column. RP-HPLC analysis of the elution fractions (Table 1.2) of acid whey loading at 8.3 mS cm^{-1} buffer conductivity showed that they mainly contained β-lac protein (>90% of total bound proteins) with a small amount of Igs (<10%), whilst regeneration fractions mainly contained macropeptides (>70%, <10 kDa size), Igs (5–10%) and all other whey proteins (α-lac and β-lac). However, whey sample loaded at buffer conductivity >44 $mS.cm^{-1}$ showed reduced the cross-reactivity (Table 1.2) for most of these contaminants (α-lac, LF and macropeptides) but there was still a significant amount of cross-reactivity found with β-lac and macropeptides. The β-lac recovered from the peptide column contained equal amounts of β-lac variants ($\beta\text{-lac}_A$ and $\beta\text{-lac}_B$), which co-eluted with bIgG in the elution buffer B (100%). An attempt was made to separate bIgG molecules from β-lac by varying the elution conditions but β-lac could not be separated from Igs. This was different than that reported by Menegatti et al. (2012), in which the eluted IgG was free from β-lac when purification was carried out under high salt concentrations (0.5M–1M NaCl) in the equilibration buffer. Therefore, in all subsequent experiments, sample buffers with at least 44 $mS.cm^{-1}$ (0.5 M NaCl) conductivity and isocratic elution conditions were employed with buffer B (100%).

Upon the completion of hexamer peptide interactions towards pure bIgG and cross-reactivity with model milk and whey proteins, various volumes of liquid milk samples were passed through the 1 mL hexamer column in the following order: acid whey at 1 $mL.min^{-1}$ (2–20 mL, containing 0.44 $mg.mL^{-1}$ of bIgG); skim milk at 0.25–0.5 $mL.min^{-1}$ or 76–152 $cm.hr^{-1}$ (1–5 mL, containing

0.53 mg.mL^{-1} of bIgG) and colostrum at 0.25–0.5 mL.min^{-1} or 76–152 cm.hr^{-1} (2–4 mL, containing 5.8 mg mL^{-1} of bIgG).

3.2 Acid whey

Frontal analysis of all acid whey loading experiments in this work was quantitatively (RP-HPLC) and qualitatively (SDS-PAGE) estimated as described in the methods Section 2.2. During the standard acid whey sample loading experiments with 10 mL and 21 mL of sample (0.44 mg.mL^{-1} of bIgG) onto 1 mL peptide column, the total amount of bound IgG was approximately 2.6 and 3.1 mg.mL^{-1} of resin, respectively. Total bound bIgG accounted for only 60% and 33% (respectively) of the total IgG loaded (acid whey) onto the column and most IgG adsorption occurred during the first 4–6 mL of acid whey loading. For these experiments, bIgG purity in the elution fractions was 55% (Table 2) with major contaminants of β-lac (variants A and B). On the other hand, regeneration fractions contained a small fraction of bIgG (<25% of total protein in the regeneration) and high amounts of macropeptides (>75%), with negligible amounts of whey proteins. However, IgG adsorption was greatly improved by loading bIgG-spiked (5 mg.mL^{-1}) acid whey samples and the contaminants binding to the resin were reduced. Frontal analysis of the bIgG-spiked experiment showed (Table 2) that most of the IgG present in the sample were bound to the resin with a leakage of <20%. For example, 1 mL of bIgG-spiked acid whey sample (5 mg.mL^{-1} bIgG) loading resulted in a binding of >80% of IgG present in the sample and the total amount of recovered bIgG accounted for 4.03 mg (Table 2). An RP-HPLC analysis of selected elution fractions of bIgG-spiked acid whey loading is given in Table 2. The purity of bIgG for this experiment was 80%, which was approximately 25% higher than that for standard acid whey sample loading experiment. These results indicate that the peptide resin binding capacity and recovered bIgG purity was depended on the IgG concentration in the feed samples.

The low recovery and purity of bIgG from standard acid whey (0.44 $mg.mL^{-1}$ of bIgG) could be because of the high amount of β-lac (3–4 $mg.mL^{-1}$) in feed samples and its cross-reactivity with hexamer peptide. Figure 2 shows the SDS-PAGE analysis of typical chromatography runs for standard acid whey sample (lanes 3–6) and bIgG spiked acid whey sample loadings (lanes 8–10). Lane 2 represents the initial acid whey (1X) sample contains major bands of α-lac (12 kDa), β-lac (16 kDa & 35 kDa), BSA (66), and bIgG (160 kDa). Lanes 3–6 (lane 4 being the main elution fraction) were elution fractions of acid whey run (10 mL) and bands at 160, 75, 50 & 25 kDa correspond to bIgG and its fragments (similar fragments were found for commercial bovine IgG sample). Lane 8–10 (Lane 9 was the main elution fraction) were the elution fractions of bIgG spiked acid whey loading run at 84 $mS.cm^{-1}$. In all elution fraction (Lanes 3–6, 8–10 & 13–14), along with bIgG, there were two other bands at 12 and 16 kDa which corresponds to two major whey proteins (α-lac & β-lac), with β-lac as the main contaminant.

3.3 Skim milk

Skim milk samples (conductivity adjusted to 84 $mS.cm^{-1}$) were passed through 1 mL peptide column at a flow rate of 0.2 $mL.min^{-1}$. To avoid column clogging and back-pressure limitations, only 4.5 mL of skim milk loaded. Similar to the acid whey results, most of the bIgG present in the applied standard skim milk (0.53 $mg.mL^{-1}$ of IgG) was bound to the peptide resin and bound IgG was successfully recovered in elution with 0.2 M sodium acetate, pH 4.0. The purity of the elution fraction of standard skim milk loading experiment was <50% (Table 2) and the total amount of recovered bIgG was 1.94 mg, which was approximately 80% of the total IgG present in the feed sample. The major contaminant (Table 1.2) in elution was β-lac, which accounted for almost 30–40% of the total proteins present in elution, and this was accompanied by other minor contaminants (caseins, α-lac and macropeptides). Regeneration fractions mainly contained

caseins and macropeptides, with a small fraction of bIgG. Figure 3 represents the SDS-PAGE analysis of standard skim milk sample loading experiments through a 1 mL hexamer peptide column at a buffer conductivity of 84 $mS.cm^{-1}$. Lane 2 of Fig. 3 represents the standard skim milk (5X) and Lanes 3–6 represent flow through fractions (2 mL fraction size). These flow through fractions (depletion of bIgG bands) indicate that almost all IgG present in the feed sample was bound to the resin. On the other hand, Lanes 7–10 (Lane 7 being the main elution fraction) show the elution fractions (1 mL fraction size) and it contained bands of different bIgG fragments (160, 75, 50 and 25 kDa) along with other bands of contaminants such as caseins, β-lac and α-lac proteins. The purity of IgG in the elution fraction was approximately 45–50% (quantitatively estimated using RP-HPLC (Table 2)). This low IgG purity (45%) in the elution is mainly due to the low concentration of bIgG (0.5–0.6 $mg.mL^{-1}$) in standard skim milk. Similar to acid whey experiments, an bIgG-spiked skim milk (5 $mg.mL^{-1}$) loading experiment resulted in better purity than with a standard skim milk feed. In Figure 3, Lanes 11 & 12 represents flow through fractions (2 mL fraction size) of the 1 mL bIgG spiked skim milk loading experiment. Bound bIgG recovered in elution (Lanes 13 & 14 in Fig. 3) had an estimated purity of 78% (Table 2). Contaminants in this fraction included minor amounts of β-lac and caseins. From the above results, it is clear that the major contaminant in elution was β-lac for both skim milk and acid whey loading experiments. For standard skim milk loading, the peptide resin showed cross-reactivity towards casein proteins along with α-lac, β-lac and macropeptides. The bound caseins were removed during regeneration were responsible for the low bIgG binding capacity of the peptide column.

3.4 Colostrum

The HWRGWV peptide resin was then tested for purification of bIgG from colostrum. Various volumes (1–4 mL) of colostrum containing 5.8 mg mL^{-1} of bIgG were loaded through a 1 mL peptide column at a flow rate of 0.5 $mL.min^{-1}$. During all loading experiments, most of IgG present in feed samples (>90%) bound to the column with a minor leakage (<10%) in flow through fractions. The chromatogram of bIgG purification from colostrums is shown in Fig. 4. The concentration of bIgG in the chromatographic fractions was determined by SPR assay as described in Section 2.2.4. The maximum column binding capacities for different loaded volumes of colostrum were estimated from mass balance calculations for individual experiments (Table 2). The elution and regeneration fractions were assayed using quantitative RP-HPLC to determine the purity of IgG in the fractions. RP-HPLC analysis of selected elution fraction (solid line, Fig. 5) suggests the fraction had >80% bIgG purity (Table 2) with the main contaminant being β-lac. Conversely, the regeneration step (dotted line, Fig. 5) indicates several protein peaks (κ-caseins, α-lac, β-lac and macropeptides) in addition to bIgG. This was confirmed by SDS-PAGE, with Figure 6 being a typical example of two consecutive colostrum loading experiments through a 1 mL peptide column. Lane 3 in Fig. 6 represents the initial protein composition of colostrum at five times dilution and Lanes 4–6 represent flow through fractions of a typical 2 mL colostrum loading at a sample buffer conductivity of 84 $mS.cm^{-1}$. Lanes 8–9 and 11–12 represent the elution fractions of two consecutive runs, respectively. All bands in Lanes 8–13 of Fig. 6 represent the expected molecular weights (160, 75, 50 and 25 kDa) of IgG. Elution fractions of these experiments have shown >80% bIgG purity (Table 2). The high purity obtained with colostrum as compared to standard skim milk and acid whey (Table 2) can be attributed to the higher concentration of IgG in colostrum (5.8 mg mL^{-1}).

Finally, the effect of buffer conductivity on bIgG interactions and cross-reactivity with all other milk proteins on the peptide column were explored using colostrum samples and the corresponding results are presented in Fig. 7. To minimize cross-reactivity and maximize IgG adsorption on the peptide resin, 2 mL of colostrum samples (for each injection) were loaded onto 1 mL peptide column at different buffer conductivities (44–160 mS cm^{-1}) while the buffer pH was kept constant at 7.4. For example, colostrum loading at a buffer conductivity of 160 mS cm^{-1} showed 55% higher bIgG binding capacity (6 mg bIgG per mL of peptide resin) than colostrum loading at 44 mS cm^{-1} (0.5 M NaCl) (3.9 mg IgG per mL of resin). In addition to the improved binding capacities, high buffer conductivity also enabled improved purity (3% higher in elution) and >70% reduction in cross-reactivity of contaminants.

However, increased buffer conductivities (160 mS cm^{-1}) decreased slightly the recovery of IgG in the elution fraction. At high salt concentrations the hydrophobic component of peptide-IgG affinity interactions is enhanced leading to increase in strength of binding, as a result of which elution buffer below pH 4 are required to ensure complete recovery of bound IgG.

3.5 Peptide interactions towards bovine IgA

This work was extended to measure hexamer peptide binding of bovine IgA. Five hundred micro litres of pure bovine IgA (0.5 mg mL^{-1}) was injected at prescribed buffer conditions (conductivity of 84 mS cm^{-1}, pH 7.4) onto 1 mL hexamer column at a flow rate of 1 mL min^{-1}. Most of the IgA (>90%) present in the sample was bound to the column with only minor leakage in flow through and bound IgA could be recovered (>95%) in elution with 0.2 M sodium acetate buffer, pH 4.0 (Table 1.1). Further interactions of bovine IgA with the peptide column and the influence of process parameters (static & dynamic adsorption capacities, cross-reactivity and buffer conductivity) were not investigated in this paper due to limited resources. However,

previous investigations related to hexamer peptide interactions with all classes of human Igs (Haiou, 2008; Liu et al., 2012; Menegatti et al., 2012; Yang et al., 2009) and current results with bovine Igs (IgG and IgA) together provide evidence of their capability to extract various classes of Igs from milk (present results) and complex fluids (Haiou, 2008; Yang et al., 2005).

4 Conclusions

A hexamer peptide resin was successfully applied for the isolation of bovine IgG from liquid milk samples, including acid whey, skim milk and colostrum. The hexamer column showed specific interactions with bovine IgG, which was successfully isolated from various liquid milk samples under suitable conditions. Resin dynamic binding capacities, purities and recoveries of IgG were greatly dependent on binding buffer conductivity and concentrations of IgG in feed solutions. The major contaminant found in the elution fractions was β-lac, which could not be separated from IgG by adjusting elution conditions. However, the interference of β-lac was greatly suppressed (>70%) by a high IgG concentration in the feed solutions (IgG-spiked milk or colostrum). The peptide resin used in this study was synthesized by the solid phase peptide synthesis (SPPS) of peptide directly onto the polymethacrylate based Toyopearl resin, which is not suited for SPPS. Recent studies in Carbonell's laboratory have revealed a significant population of truncated peptides on the resin, which might be responsible for the non-specific binding of β-lac observed in this study. To address this issue, work is being carried out to couple the pure peptide (obtained by synthesis on regular SPPS resin) to chromatographic resins. Preliminary results have indicated reduced non-specific interactions and high IgG capacity (65 mg/ml). The detailed results will be published on the completion of work.

Unlike Protein A/G, the HWRGWV resin also showed interactions with bovine IgA, similar to human IgA, which indicates that this peptide resin has the potential to isolate both classes of

bovine Igs. The present investigation along with previous work (Yang et al., 2009) using this peptide to isolate Igs from human serum, mammalian cell culture broths and bovine milk samples indicate that this peptide is a potential ligand for extracting various classes of Igs and could be a possible alternative to existing affinity chromatography ligands (Protein A/G).

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The authors have declared no conflict of interest.

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Table 1

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Table 1.1: Summary of Igs bound and recovered from pure IgG and IgA loading experiments and standard acid whey loading experiments at different buffer conductivities. RP-HPLC method was used here to estimate IgG concentration in various acid whey samples and chromatography fractions. IgG concentration of pure IgG experiments were measured by RP-HPLC method.

Pure IgG Samples	Volume (mL)	Total IgG (mg) Bound	Recovered IgG (mg) Elution	Recovered IgG (mg) Regeneration	% IgG Bound
IgG (1 mg.mL^{-1})	6	4.8±0.20	4.3±0.09	0.5±0.06	80
IgG (5 mg.mL^{-1})	1	4.6±0.12	4.2±0.07	0.4±0.09	92
IgG (9 mg.mL^{-1})	2	11.8±0.03	11±0.05	0.8±0.09	65
IgA (0.5 mg.mL^{-1})	*0.5*	*0.23*	*0.21*	*0.02*	92
Acid Whey sample conductivity (mS.cm^{-1})					Purity (%)
8.3	10	0.7±0.10	0.5±0.10	0.2±0.09	2.3
44	10	1.8±0.05	1.5±0.03	0.3±0.08	36
84	10	2.6±0.05	2.0±0.10	0.5±0.12	52
120	10	2.9±0.09	2.1±0.05	0.8±0.20	53
160	10	3.2±0.05	2.2±0.12	1.1±0.15	60

Table 1.2: Hexamer peptide ligands interaction/cross-reactivity with various samples of whey and milk proteins. Percentage of protein bound to the peptide column under defined sample conditions are given in the table. RP-HPLC analysis alone was used here for quantifying various whey proteins of acid whey experiments. SPR and RP-HPLC methods were used for quantifying of whey proteins of skim milk and colostrum experiments. SDS-PAGE was used for qualitative analysis of various samples.

Sample	α-Lac (%)	β-Lac (%)	BSA (%)	Caseins (%)	Macro-peptides (%)	LF (%)	IgG (%)
Acid Whey (10 mL) (8.3 mS.cm^{-1})	5.36	91.05	0.00	0.00	0.53	0.00	3.06
Acid Whey (10 mL) (44 mS.cm^{-1})	4.44	53.89	0.00	0.00	5.23	2.89	33.54
Acid Whey (10 mL) (84 mS.cm^{-1})	0.79	42.62	0.00	0.00	5.13	1.44	50.02
Skim milk (4.5 mL) (84 mS.cm^{-1})	1.66	31.24	0.00	15.99	0.26	0.00	50.85

Table 2

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Table 2: Summary of total Igs bound and recovered in each elution and regeneration step, along with their purities from different liquid milk samples at different binding conditions. The terms used in this table are AW-acid whey, AW*-IgG spiked acid whey; SM-Skim Milk and SM*-IgG spiked Skim Milk; CSM-Colostrum Skim Milk at conductivity of 44 $mS.cm^{-1}$, CSM*- Colostrum Skim Milk at conductivity of 84 $mS.cm^{-1}$ and CSM**-Colostrum Skim Milk at conductivity of 160 $mS.cm^{-1}$. Buffers were all constant at pH 7.4. RP-HPLC analysis method was only used for quantifying various whey proteins of acid whey experiments. SPR and RP-HPLC (where suitable) methods were used for quantifying of whey proteins of skim milk and colostrum experiments. SDS-PAGE was used for qualitative analysis of various samples.

Sample	Volume (mL)	Total Bound (mg)	Elution recovered (mg)	Purity (%)	Regeneration recovered (mg)	Purity (%)
AW	10	2.6±0.02	2.1±0.05	52±0	0.51±0.04	N/A
AW	21	3.1±0.06	2.3±0.05	55±1	0.88±0.01	N/A
AW*	1	4.0±0.00	3.2±0.00	80±0	0.81±0.00	N/A
SM	4.5	1.9±0.01	1.17±0.04	45±1	0.76±0.03	N/A
SM	1	0.4±0.00	0.26±0.01	40±1	0.13±0.01	N/A
SM*	1	3.0±0.06	2.6±0.04	78±2	0.39±0.02	N/A
CSM	2	3.9±0.03	2.49±0.07	81±1	1.38±0.08	32
CSM*	2	4.6±0.03	3.2±0.14	85±1	1.46±0.11	52
CSM**	2	6.0±0.11	4.0±0.02	86±1	2.04±0.13	56

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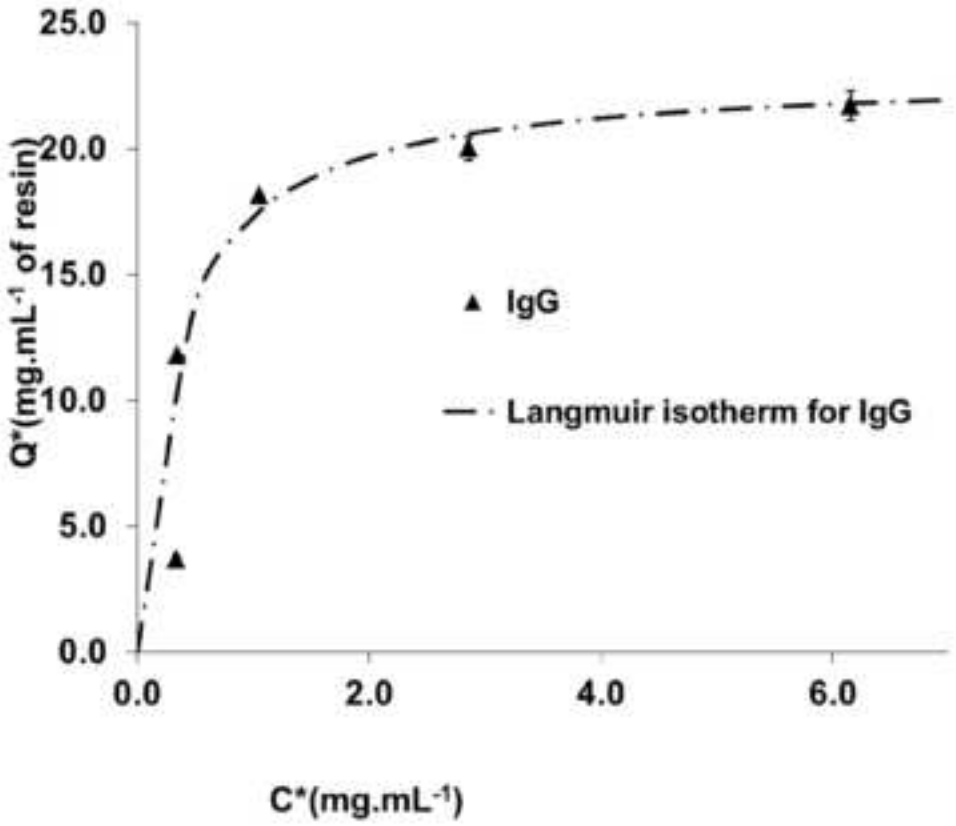

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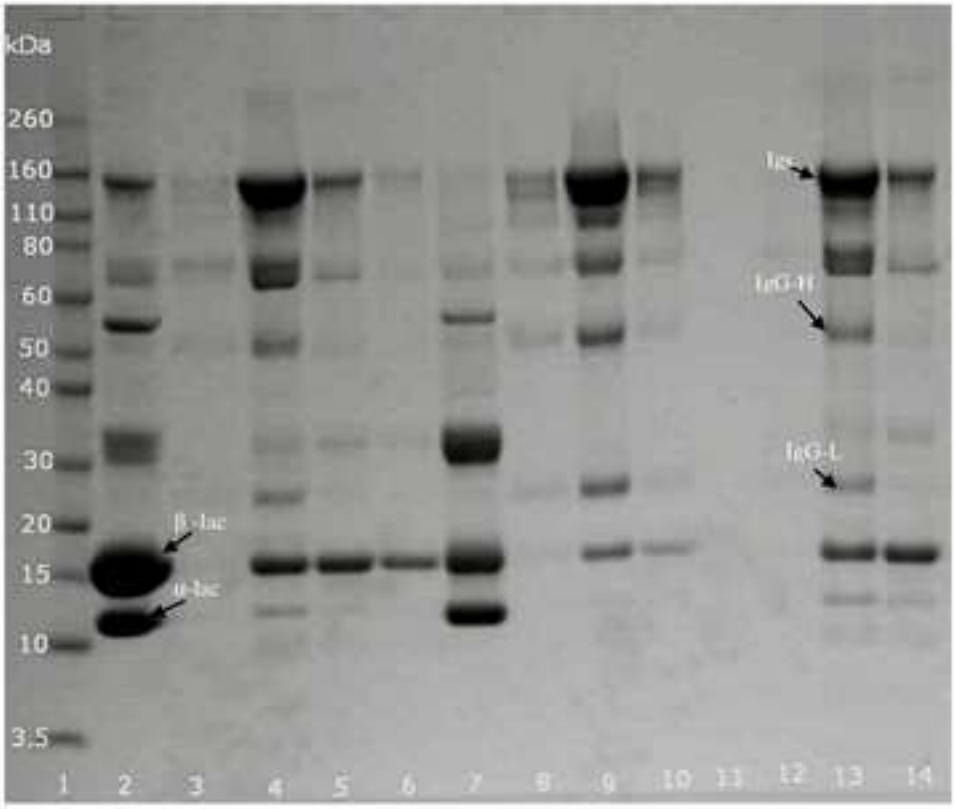

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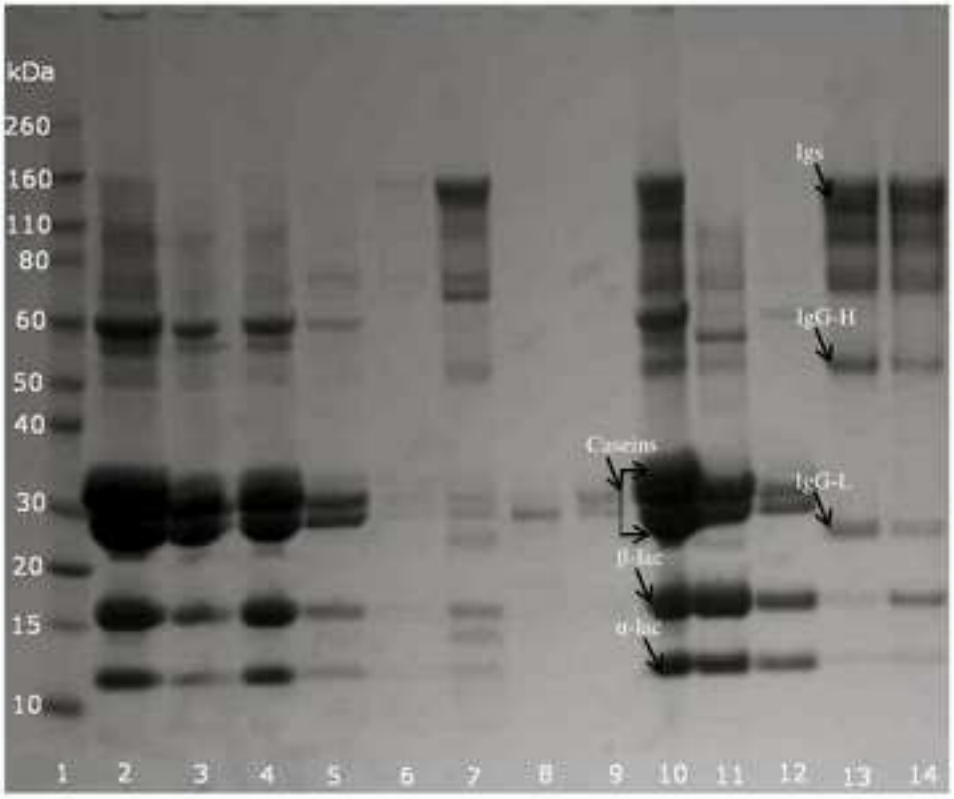

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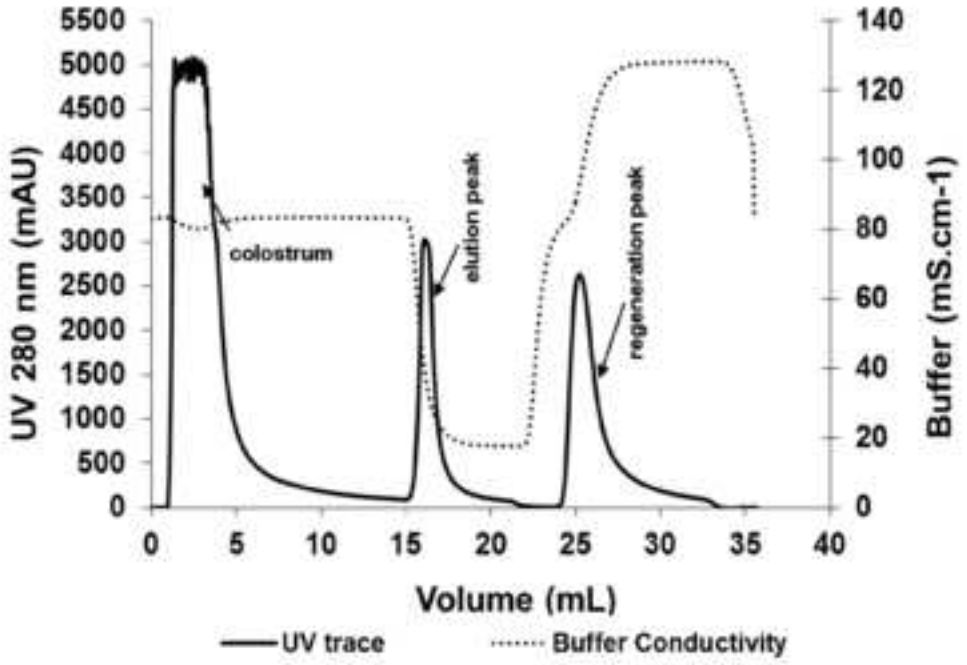

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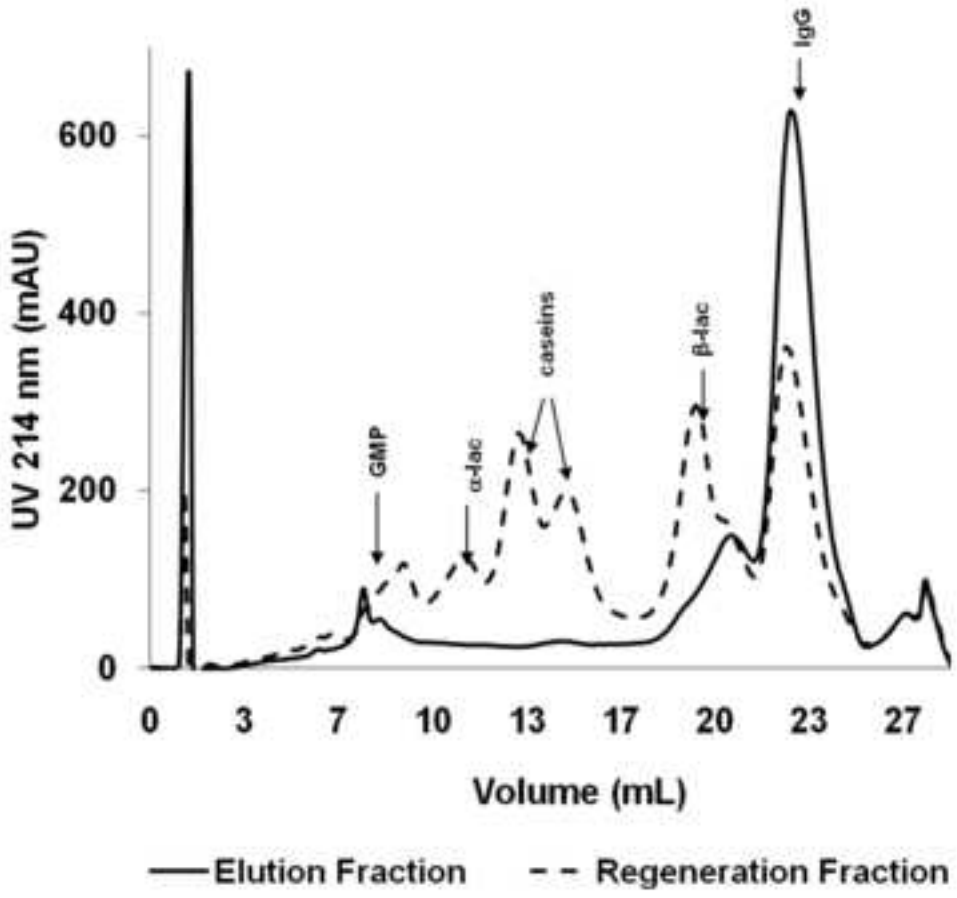

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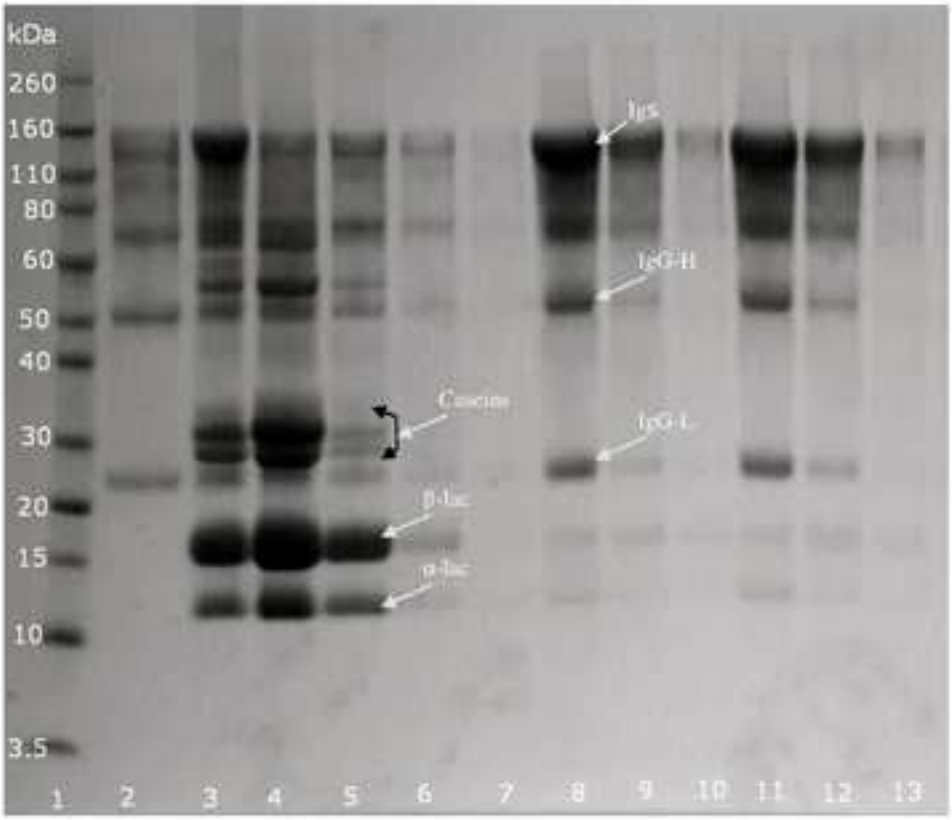

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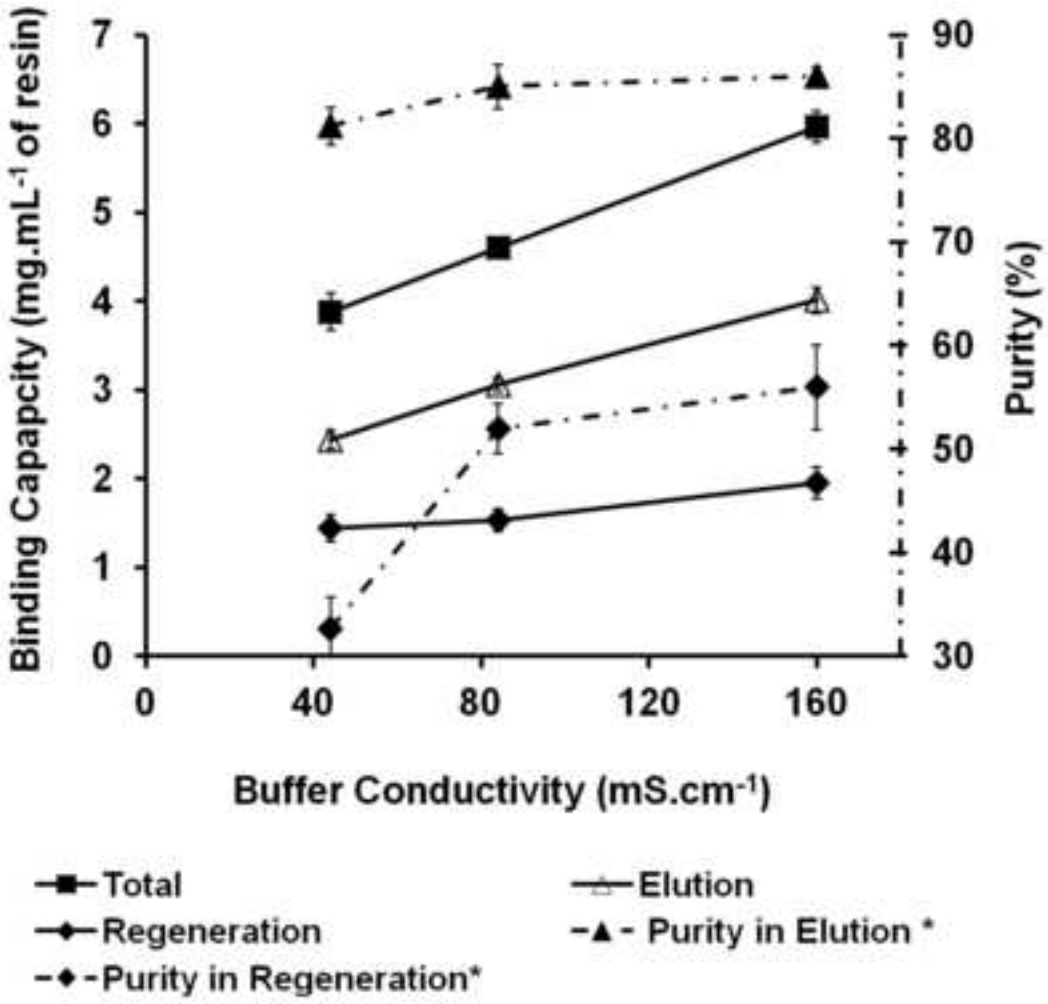

Figure Legends:

Figure 1: Equilibrium binding of IgG measured in a standard solution at binding buffer conductivity of 84 mS.cm^{-1} and pH 7.4. The line fitted through the points corresponds to the Langmuir isotherm (K= *0.33 mg.mL*$^{-1}$) and Q_o = *22.99 mg.mL*$^{-1}$ of bovine IgG).

Figure 2: SDS-PAGE (4–12%) analysis of whey sample loading experiments set 2. **L1**: protein ladder; **L2**: initial whey (1X); **L3-6**: elution fractions of acid whey sample loading (10 mL) at buffer conductivity of 84 mS.cm^{-1}; **L7**: initial whey (5X); **L8-10**: elution fractions of IgG spiked (5 mg.mL^{-1}) whey sample (1 mL) loading at buffer conductivity of 84 mS.cm^{-1}; **L11-14**: elution fractions of acid whey sample (10 mL) loading at buffer conductivity of 160 mS.cm^{-1}.

Figure 3: SDS-PAGE (4–12%) analysis of skim milk sample loading experiments at sample buffer conductivity of 84 mS.cm^{-1}. **L1**: protein ladder; **L2**: initial skim milk (5X); **L3-5**: flowthrough fractions of skim milk loading (4.5 mL) run; **L6-9**: elution fractions of corresponding run; **L10**: initial skim milk spiked with IgG (5X); **L11-12**: flowthrough fractions of IgG spiked skim milk loading (1 mL); and **L13-14**: elution fractions of corresponding run.

Figure 4: UV trace (280 nm) of a colostrum (2 mL, 84 mS.cm^{-1}) sample loading into the 1 mL hexamer peptide column at a flow rate of 0.5 mL.min^{-1}. Arrows indicates the application of sample loading, elution and regeneration buffers, and their corresponding peaks.

Figure 5: RP-HPLC analysis of selected elution and regeneration fractions of colostrum sample (2 mL) loading at 84 mS.cm^{-1} chromatography experiment (Fig. 4).

Figure 6: SDS-PAGE (4–12%) analysis of colostrum sample loading experiments at a sample buffer conductivity of 84 mS.cm^{-1}. **L1**: protein ladder; **L2**: pure bovine IgG; **L3**: initial colostrum (5X); **L4-6**: flowthrough fractions of colostrum (2 mL) loading run1; **L7-10**: elution fractions of corresponding run; **L11-13**: elution fractions of colostrum (2 mL) loading run 2.

Figure 7: Hexamer peptide resin binding selectivity towards Igs of colostrum in various buffer conductivities. In this figure, X-axis indicates the buffer conductivity (44–160 mS.cm^{-1}). Primary Y-axis indicates the total bound Igs and amounts of Igs recovered in both elution and regeneration steps. Secondary Y-axis represents the purity of elution and regeneration fractions. Dotted lines indicate the purity data and solid lines indicate the capacity data.