

# A 3-mechanism model for adsorption of IgG on CHT™ ceramic hydroxyapatite

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## Introduction

Previous work with IgG discussed interactions of protein molecules by a combination of two mechanisms: phosphorylation and charge-charge interactions. Phosphorylation involves the interaction of negatively charged amino groups on proteins with the positively charged calcium groups on CHT, and calcium metal affinity, which involves the protein carboxyl groups and the positively charged calcium groups on CHT. Although the initial binding of the latter mechanism is also electrostatic, it eventually forms much stronger coordination bonds. Phosphorylation involves the interaction of negatively charged amino groups on proteins with the positively charged calcium groups on CHT. Metal affinity ions such as calcium, as presented in CHT, can act as electrophiles, seeking the possibility of forming electron pairs with other atoms so that a bond or charge-charge interaction can be formed. Such an interaction has been demonstrated between metal ions and the imidazole groups of histidyl side chains in a variety of enzymes. Extension of this observation to IgG which carries six histidine residues on its FC region and other histidine residues on its CDR regions is being investigated.

The present study attempts to determine the relative contributions of the three mechanisms: phosphorylation, charge-charge interaction, and calcium-histidine interaction. Results will be presented demonstrating the effect of pH, phosphate and imidazole on purification performance.

## Experimental

**Chemicals**  
 Protein A eluate, Clarified tissue culture fluid obtained from Avicel Bioscience (Tustin, Ca) was applied to a Millipore column (BioSieve™ 3000) and purified by ion exchange chromatography on a HiTrap™ SP5 column (Bio-Rad Laboratories, Hercules, Ca). The phosphate buffers were prepared with sodium phosphate monobasic in nanopure water and were titrated to target pHs with 1M NaOH.

**Protein A**  
 Protein A eluate, Clarified tissue culture fluid obtained from Avicel Bioscience (Tustin, Ca) was applied to a Millipore column (BioSieve™ 3000) and purified by ion exchange chromatography on a HiTrap™ SP5 column (Bio-Rad Laboratories, Hercules, Ca). The phosphate buffers were prepared with sodium phosphate monobasic in nanopure water and were titrated to target pHs with 1M NaOH.

**Column**  
 The experiments were conducted on a BioScale™ IATC column obtained from Bio-Rad Laboratories (Hercules, Ca). The column dimensions were 0.7 x 2.6 cm. The column was dry packed using a density of 0.60 gm/mL. Each column was wetted in ten column volumes of 2% ethanol before equilibration. All chromatography experiments were automated and executed using the Biologic DuoFlow™ System and software. To determine the elution profile, 0.005M Na phosphate, pH 6.5 (buffer A). After IgG injection, the column was washed with ten column volumes of equilibration buffer. The IgG was eluted using a gradient in NaCl concentration to 1.5M (buffer B). This was achieved by programming the chromatography system to go from 0 to 100% B in 40 column volumes. The column was cleaned with 0.3M NaOH, phosphate pH 7 prior to equilibration before each run. For imidazole containing buffers, imidazole was added to 0.3M NaOH, phosphate pH 7 and the pH titrated with 8N HCl.

**Resolution factor (R)**  
 The resolution factor reflects the quality of separation between the monomer (A) and the aggregates (B). It is determined according to the equation,  $R = 2(t_B - t_A)/(W_A + W_B)$ , where t and W correspond to the retention time and peak width, respectively. A resolution value of 1.5 implies a complete separation of the two adjacent peaks.

**Peak of monomer**  
 It is located by determination of % B in which the monomer's eluted peak maximum appears.

**Frontal dynamic binding capacity (DBC)**  
 Frontal loading studies to 10% breakthrough for CHT was performed at 600 cm/hr. After equilibration, each column was loaded with a constant volume of 1.5 column volumes of monomer solution. The breakthrough was determined at 200 nm for a polyclonal antibody solution with an OD value of 1.2. The antibody solution was loaded onto the column through the injector valve after attaining 100% breakthrough. DBC at 10% breakthrough was calculated by multiplying the antibody concentration in the lead by the frontal volume.

**Statistical Factorial Design (SFD)**  
 The screening experiment was designed to help predict what results are expected when process parameters are changed. The screening experiment was designed with two levels of pH 6.5 and 7.5; two levels of phosphate concentrations: 0.005M and 0.02M, and two levels of imidazole concentrations: 0M and 0.3M. The experiments were arranged in 23 statistical factorial design with a total of eight runs. These runs using the center point conditions were also carried out. These runs are important to establish linearity trends and to estimate errors in the model.

Concentrations (factor 2) and imidazole concentrations (factor 3) on resolution and peak elution were explored using Unmetrics MODDE 7 software package.

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Figure 1: DBC optimum at pH 6.7 to 6.8

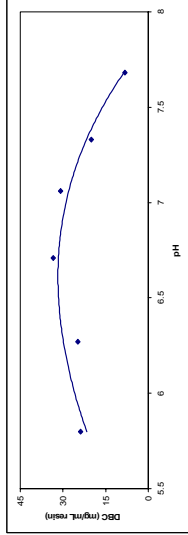


Figure 2: Inverse relation between DBC and PO<sub>4</sub> concentration

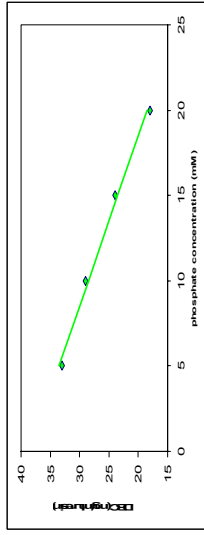


Figure 3: DBC decreases as imidazole concentration increases

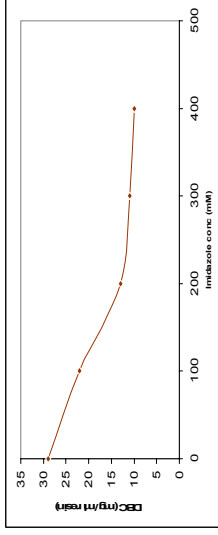


Figure 4: DBC dramatically decreases at 150 mM NaCl

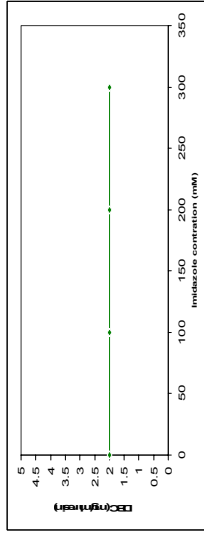
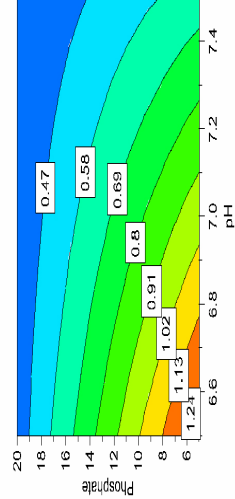


Figure 5: Inverse effect of pH and PO<sub>4</sub> on resolution



## Factorial Design Analysis

Run	pH	Phosphate	Resolution
1	7.4	20	0.5
2	6.8	20	0.5
3	6.2	20	0.5
4	7.4	5	0.5
5	6.8	5	0.5
6	6.2	5	0.5
7	7.4	20	0
8	6.8	5	0
9	7.4	15	0.19
10	6.8	15	0.19
11	7.0	15	0.18
12	7.0	15	0.18



Table 1: Effect of imidazole & NaCl on migration of peak maximum

IgG <sub>1</sub>	% B at peak maximum	Shift index (%)
Control	43	0
0.12M NaCl	34	-21
0.3M imidazole	31	-28
Control	48	0
0.12M NaCl	42	-13
0.3M imidazole	32	-33

Table 2: SFD Results and Conclusions

Resolution	Location of peak maximum
Improved at pH 6.5	Higher eluent concentration at pH 6.5
Improved at 5 mM phosphate	Higher eluent concentration at 5 mM phosphate
No improvement in majority of the cases containing 0.3M imidazole	Higher eluent concentration without imidazole