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Use of Hydrophobic Interaction Chromatography With a Non-Salt Buffer System for Improving Process Economics in Purification of Monoclonal Antibodies

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Abstract

One of the barriers to more prevalent application of hydrophobic interaction chromatography (HIC) for purification of monoclonal antibodies is that the sample often elutes at salt concentrations that require buffer exchange before the next purification method can be performed. The selectivity provided by HIC is often so powerful that the technique is used despite these limitations, but residual salts constrain process design nevertheless. This report describes a non-salt-based (NSB) binding buffer system that mimics the selectivity of salt-based HIC buffers, but does so at conductivities directly compatible with ion exchange chromatography (IEC). This makes it possible to apply HIC fractions directly to IEC without intermediate buffer exchange, greatly extending the flexibility of HIC with respect to sequential placement in multi-method purification schemes. Results are shown illustrating practical application of the NSB system to multi-step purification of monoclonal antibodies.

Introduction

Hydrophobic interaction chromatography has been traditionally classified as a "high-salt" technique, however this classification is not explanatory in terms of the mechanism by which salts promote binding. Several investigators have noted that precipitating salts are excluded from protein surfaces. (1-3) This leaves a pure water hydration sheath surrounding the protein. The thermodynamic discontinuity between the pure water sheath and the high-salt bulk solution creates an exclusionary pressure so that when pure-water-hydrated proteins encounter other such proteins, their association is stabilized. Given the presence of a preferential binding substrate in the system, such as a hydrophobic stationary phase, proteins associate instead with the column.(3) The higher the salt concentration, the more strongly the proteins are excluded from the mobile phase, and the stronger their association with the stationary phase.

The amino acid glycine has been shown to be excluded from protein surfaces in a manner similar to precipitating salts.(1,2). Although not widely regarded as a protein precipitant, it has been used to efficiently precipitate fibrinogen.(4) Glycine has also been reported to enhance protein retention on HIC columns of weak-to-moderate hydrophobicity.(5) The degree of enhancement on these columns is not sufficient to promote retention independently, but more recent results show that glycine is able to support dynamic capacities greater than 25mg IgG /mL of gel on Phenyl columns.(6) Mole per mole, glycine is a far less effective binding promoter than the usual HIC salts such as ammonium sulfate, but it features electrostatic characteristics that make it much more compelling from a process perspective. Between the pKas of its carboxyl (2.35) and amino (9.76) groups, glycine is zwitterionic and contributes nothing to conductivity.(7) This allows HIC fractions to be applied directly to ion exchangers with little more than pH titration of the sample, thereby eliminating more costly intermediate sample equilibration steps such as buffer exchange chromatography or diafiltration.(6)

The only limitation of glycine in comparison to traditional HIC salts is its relative cost. In previous work, crude sample was equilibrated by addition of glycine to a concentration of 2.5M.(6) The column was equilibrated in the same buffer, and then washed with the same buffer after sample application. Still more glycine was consumed in the elution gradient. In the present study, the sample is equilibrated by direct addition of sodium chloride to a concentration of 2.0M. The column is equilibrated and washed with the 2.0 M sodium chloride buffer, then the system is switched to 2.5 M glycine, and the elution gradient is applied. This scheme conserves the advantages of low conductivity elution while substantially reducing scale-up process expense. To demonstrate the ability of NSB-HIC to improve purification process continuity, a three-step purification is illustrated, beginning with filtered ascites, first-step cation exchange, second-step NSB-HIC, and final-step anion exchange chromatography.

Results and Discussion

The results of this study demonstrate the ability of NSB-HIC to support effective multi-method IgG purification procedures, without requirement for intermediate sample preparation steps such as buffer exchange chromatography or diafiltration. Since compatibility of HIC fractions with downstream ion exchange steps was a primary objective of this study, the inclusion of 0.05M sodium chloride in the eluting buffer requires explanation. Simply put, IgG solubility is depressed in the complete absence of salts, and it elutes in a smear rather than a sharp peak. Chromatograms for the three purification steps are illustrated in **Figures 1–3**. A reduced SDS PAGE gel of selected fractions is illustrated in **Figure 4**.

The implications for purification process economics are substantial. **Table 1** illustrates some of the cost factors associated with intermediate processing steps. In addition to placing an unnecessary burden on process development and validation, these factors inflate the production cost of every lot of purified antibody that is subsequently manufactured. Even to the extent that glycine is more expensive than traditional HIC salts, the compensatory benefits of eliminating intermediate steps are overwhelming.

Table 1. Cost Factors Associated With Intermediate Sample Equilibration by Buffer Exchange Chromatography, Ultrafiltration and Diafiltration

- Direct expense of hardware
- Direct expense of media
- Process development expenses (method specifications, cleaning, cycle limits, storage)
- Validation expenses (method specifications, cleaning, cycle limits, storage)
- Documentation expense (SOPs, process records)
- Product losses (direct adsorption, internal hold-up volumes, other inefficiencies)
- Frequent formation of aggregates (diafiltration)
- Additional process time (reduces facility throughput/capacity)
- Increased process variability coincident with increased process complexity
- Additional opportunities for process/operator errors
- Equipment and media storage during non-use periods

The combination of NSB-HIC with ion exchange chromatography supports other immediate and long-term economic benefits as well. Protein A and protein G affinity chromatography are widely used for antibody purification despite their grossly disproportionate expense. This is largely due to the absence of more economical generic alternatives. This and other studies have shown that two-step NSB-HIC/ion exchange purifications can consistently produce antibody at a purity level sufficient for diagnostic applications or for animal studies.(6,8) This study and ongoing work indicate that three-step NSB-HIC/ion exchange purifications can likewise fulfill purification needs for later clinical studies and commercial manufacturing.

NSB-HIC/ion exchange puri-fication methods promise far greater benefits over affinity methods in other ways. The antibody industry is becoming increasingly aware that long overlooked features of affinity purification seriously complicate both process development and validation, and even threaten the long-term commercial success of affinity purified products. **Table 2** summarizes some of the nearly 200 publications that describe immunotoxic effects of both protein A and protein G.(9) The better known denaturative effects of these methods are also serious concerns. These factors risk both product efficacy and patient health. Combined with the direct cost burden of affinity purification, they highlight the need for the industry to evolve toward purification methods that avoid these critical liabilities.

Table 2. Immunological Side Effects of Protein A and Protein G*

|	
Complement Activation	Proliferation of B lymphocytes
Accelerated IgG catabolism	
Agglutination of granulocytes	Proliferation of T lymphocytes
Leukocyte chemotaxis	
	Altered ion transport across
Histamine release from Leukocytes	lymphocyte membranes
Induction of hypersensitivity Anaphylaxis	
	Stimulation of DNA synthesis in
Migration of IgG receptors on cell surfaces	activated B lymphocytes
Macrophage stimulation	
	Induction of immunoglobulin secretion
Induction of rheumatoid factor	by B lymphocytes
Lymphokine secretion	
	Proliferation of natural killer activity
Potentiation of immune response	of human lymphocytes
Modulation of phagocyte function Platelet injury	Inhibition of aggregate binding by lymphocytes
	Inhibition of lymphocyte colony formation
*Refer to reference 9 for citations	Inhibition of binding to Fc receptors
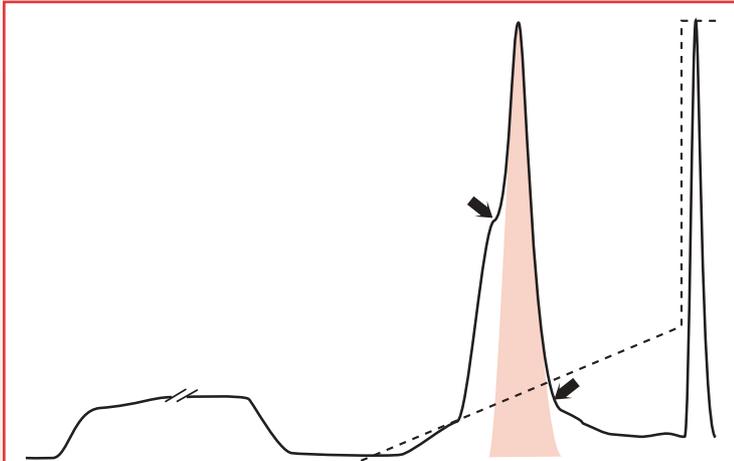
Figure 1. Cation exchange of ascites on SP-5PW-HR. Arrows indicate pool boundaries. Shaded area indicates antibody distribution. Dashed line indicates salt gradient. 3.0 AUFS. See methods section for conditions.
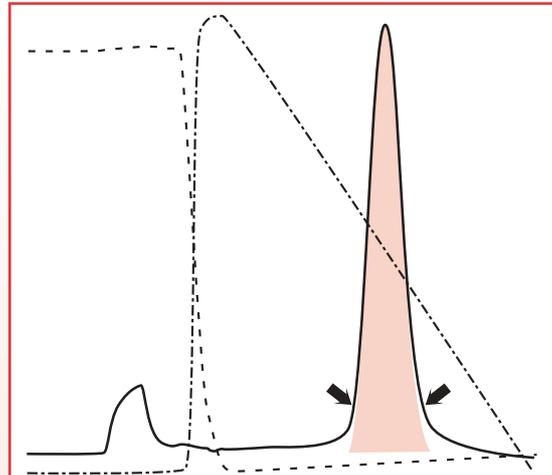

Figure 2. NSB-HIC of cation exchange pool on Phenyl-5PW-HR. Arrows indicate pool boundary. Shaded area indicates antibody distribution. Simple dashed line indicates conductivity. Double dashed line indicates glycine gradient. 1.5 AUFS. See methods section for conditions.
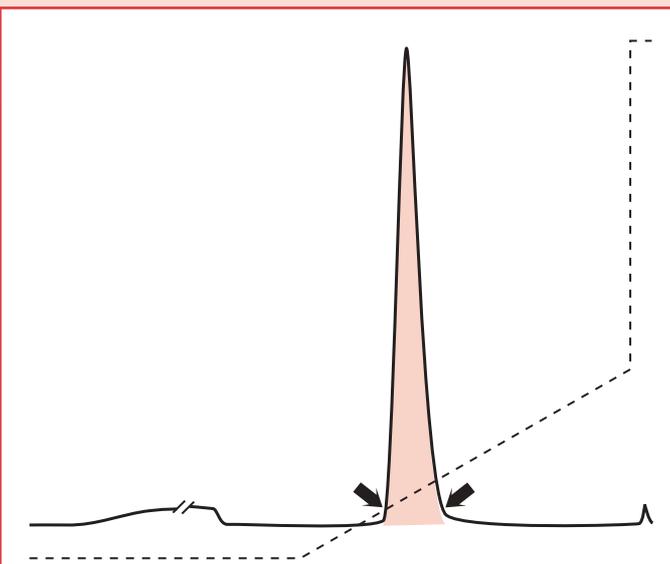

Figure 3. Anion exchange of NSB-HIC fraction on DEAE-5PW-HR. Arrows indicate pool boundaries. Shaded area indicates antibody distribution. Dashed line indicates salt gradient. 2.0 AUFS. See methods section for conditions.
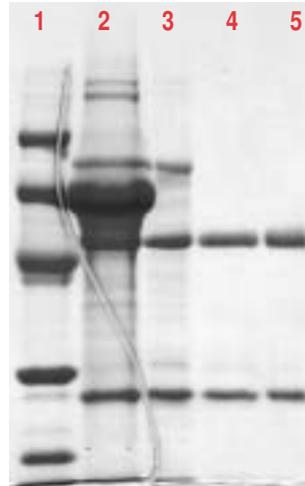

Figure 4. Reduced SDS-PAGE of purification steps.

1. Molecular weight stds.
2. Ascites
3. Cation exchange pool
4. NSB-HIC pool
5. Anion exchange pool

Acknowledgements

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Materials and Methods

Prepacked TSK MD-G Phenyl-5PW-HR, SP-5PW-HR, and DEAE-5PW-HR columns (68mm x 10mm, 5.3mL) were obtained from Tosoh Bioscience (Montgomeryville, PA). Salts and buffers were obtained from Sigma Chemical Company (St. Louis, MO). Murine IgG1 ascites were obtained from Becton Dickinson BioSciences (San Jose, CA).

Buffers were prepared with reverse osmosis/deionized water and vacuum filtered to 0.22μm before use. For the cation exchange step, buffer A was 0.05M MES, pH 5.5. Buffer B was 0.05M MES, 1.0 M sodium chloride, pH 5.5. For NSB-HIC, buffer A was 0.05M sodium phosphate, 2.0M sodium chloride, 20mM EDTA, pH 7.5. Buffer B was 0.05M Tris , 2.5M glycine, pH 7.5. Buffer C was 0.05M Tris, 0.05M sodium chloride, 10% ethylene glycol, pH 7.5. For the anion exchange step, buffer A was 0.05M Tris, pH 8.5. Buffer B was 0.05M Tris, 1.0M sodium chloride, pH 8.5.

Chromatography conditions, cation exchange step: equilibrate column with 10 column volumes (CV) buffer A at a linear flow rate of 400cm/hr. Filter 5mL ascites to 0.22 μm. Equilibrate sample pH by addition of 0.5 mL 1.0M MES, pH 5.5. Load sample by on-line dilution, 20% sample, 80% buffer A. Wash, 5CV buffer A. Elute, 10CV linear gradient to 25% buffer B. Strip, 2CV 100% buffer B. Conditions for NSB-HIC: equilibrate column, 5CV buffer A at a linear flow rate of 400 cm/hr. Equilibrate IgG pool from previous step by addition of sodium chloride to 2.0M. Load without dilution. Wash 5CV buffer A, then 2CV buffer B. Elute, 10CV linear gradient to buffer C. Conditions for anion exchange: equilibrate column with 10CV buffer A at a linear flow rate of 400 cm/hr. Equilibrate pH of IgG pool from previous step by addition of 1.0M Tris pH 8.5, 10% (v:v). Load sample by on-line dilution 50% sample, 50% buffer A. Wash, 5CV buffer A. Elute, 10CV linear gradient to 30% buffer B. Strip, 2CV 100% buffer B.

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