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Bioprocess Tutorial

Hydrophobic Charge-Induction Chromatography

Method Has Several Advantages Over Traditional Antibody Production Methods

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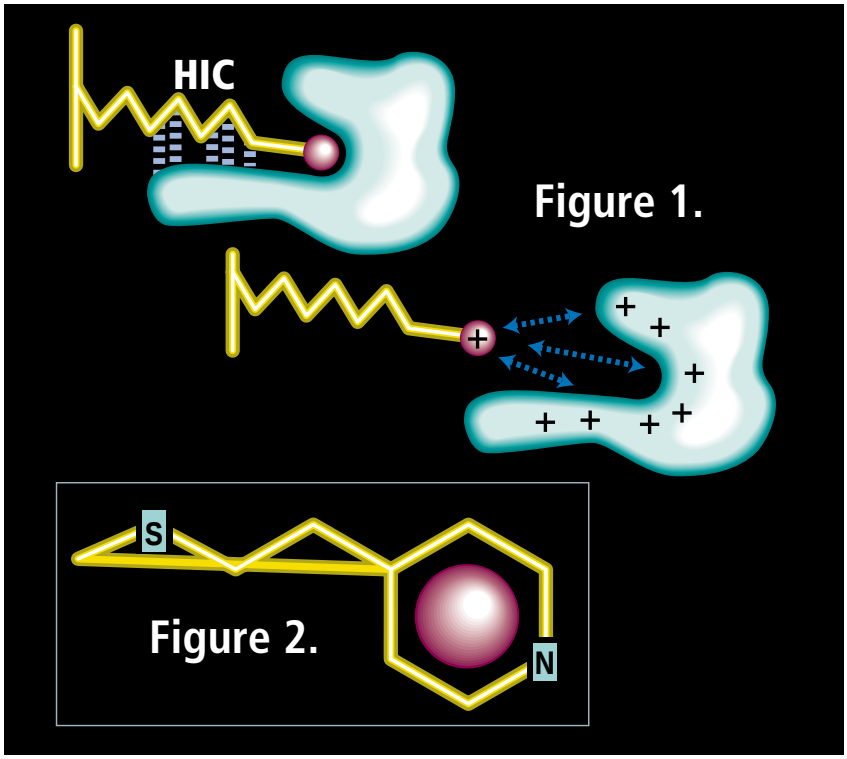

Figure 1. Mechanism for hydrophobic charge-induction chromatography. At neutral pH, (top) the ligand is uncharged and binds molecules through mild hydrophobic interaction. As the pH is reduced (bottom), the ligand becomes positively charged and hydrophobic binding is disrupted by electrostatic charge repulsion.

Figure 2. The preferential binding of antibodies by the 4-mercaptoethylpyridine ligand is further enhanced by the aliphatic chain, and the thio-ether group on the linker arm.

Hydrophobic charge-induction chromatography, or HCIC, is a novel chromatographic technique for separation of biological macromolecules, based on the pH-dependent behavior of ionizable, dual-mode ligands.

Selectivity is orthogonal to ion exchange and other commonly employed chromatographic modes (*Figure 1*). Binding is based on mild hydrophobic interaction and is achieved under near-physiological conditions, without the addition of lyotropic or other salts.

Desorption is based on electrostatic charge repulsion and is accomplished by reducing the pH of the mobile phase. Under mild acidic conditions (pH 4.0–4.5), both the ligand and target molecule take on a net positive charge; binding is thus disrupted and elution occurs. Elution is conducted using dilute buffer (e.g., 50 mM acetate).

The new BioSepra MEP HyperCel sorbent from **Life Technologies, Inc.** (LTI; Rockville, MD) has been optimized for capture and purification of monoclonal and polyclonal IgG.

The heterocyclic ligand, derived from 4-mercaptoethylpyridine (4-MEP; *Figure 2*), provides efficient capture and purification of antibodies from a broad range of sources, such as animal sera, ascites fluid and a variety of cell culture

supernatants, including protein-free, chemically defined, protein-supplemented and serum-supplemented media.

Advantages

HCIC offers a number of process advantages over more traditional methods for antibody purification, such as protein A or ion exchange:

• Sample preparation is reduced to clarification since feedstocks may be applied without adjustment of ionic strength or pH (*Figure 3*). Dynamic binding capacities of ≥30 mg IgG per ml of sorbent, at 10% breakthrough, are routinely achieved, even for murine IgG_1, without the addition of lyotropic or other salts to enhance binding (*Table 1*).

Preconcentration of dilute samples is not required since efficient capture is achieved even with feed stocks as dilute as 50–100 µg of antibody per ml.

• Gentle, pH-controlled elution, using dilute buffer, reduces the risk of antibody aggregation that may occur at lower pH, and eliminates the need for desalting or diafiltration of the IgG fraction.

• The new material is base-stable and can be easily cleaned with 1 M sodium hydroxide. In addition, low leakage of a low-toxicity ligand makes this material a good alternative to protein A for antibody production, for both in vivo and in vitro use.

pH-Dependent Binding

At pH values from 7–9, the binding capacity for human polyclonal IgG ranges from 25–33 mg/ml. As pH is reduced, binding capacity decreases, consistent with the fundamental mechanism.

Figure 3 illustrates that high binding capacity is achieved at pH values representative of typical feedstocks. Precipitation, which is sometimes observed during adjustment of feedstock pH, is thus avoided.

Table 1. MEP HyperCel Capacities

Binding capacity	mg/ml of sorbent*
Human polyclonal IgG	32
Murine monoclonal IgG_1 (from ascites fluid)	37
Murine monoclonal IgG_{2a} (from cell culture)	34

* Determined in a packed column, dynamic binding capacity determined at 10% breakthrough.

Table 2. Examples of Purifications

Feedstock	Total proteins (mg)	IgG content (mg)	IgG initial purity (%)	Final IgG purity (%)	Recovered IgG (mg)	Overall IgG yield (%)	Purification factor
CCS[1] with FBS	1,740	30	1.7	69	23	76	40
Protein-free CCS	330[2]	17	5[2]	99	17	99	19[2]
Bovine serum	440	121	28	75	105	87	3
Ascites fluid	55	9	16	83	7	79	5

[1] Cell culture supernatant
[2] BCA methodology
Working buffer, PBS; pH 7; flow rate, 70 cm/h

Figure 3

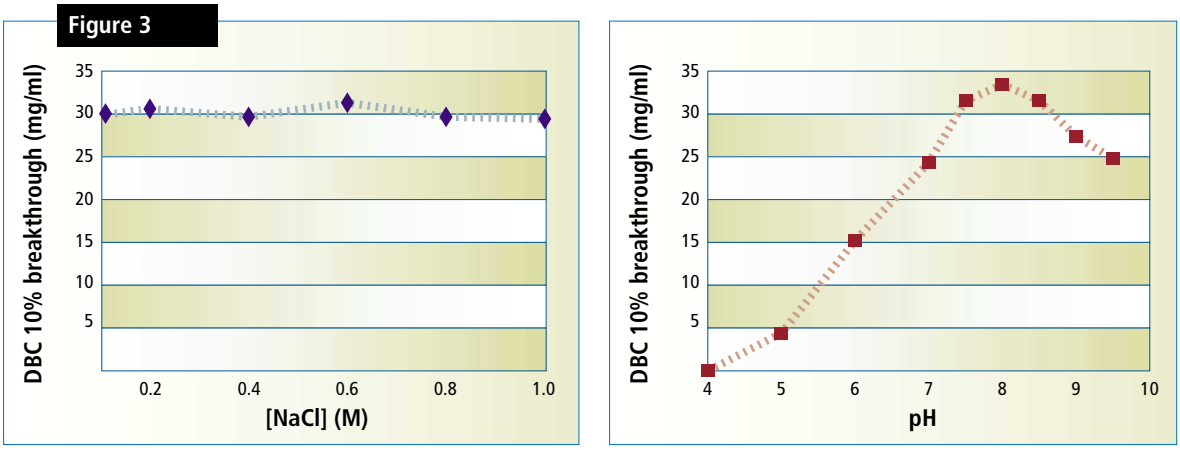

Figure 3. Influence of ionic strength (left) and pH (right) on binding capacity. IgG capacities obtained at 10% breakthrough on MEP HyperCel vs. pH and ionic strength of the binding buffer. Experimental conditions: Column 1.1 cm ID x 9 cm; Sample: IgG (2 mg/ml); Flow rate: 90 cm/h.

Independent of Ionic Strength

At neutral pH, binding capacity is nearly constant at sodium chloride concentrations ranging from 50 mM to 1 M. It is therefore possible to load many typical feedstocks without adjustment. Viscous feedstocks, e.g. animal sera, may be diluted as required.

Independent of Sample Concentration or Source

No significant variation in capacity is observed with samples ranging in concentration from 50 µg to 5 mg IgG/ml. Thus, no preliminary concentration of feedstock is required: Concentration and initial purification can be achieved in a single step.

When antibodies are isolated from protein-free cell culture supernatant, product 90% pure (SDS-PAGE) is typically obtained. Product of 99% purity was recovered in a single chromatographic step (*Table 2*). Isolation from crude feedstock (bovine serum and ascites fluid or cell culture supernatant supplemented with FBS) is also illustrated, with purity values ranging from 69–83%.

When antibody is isolated from albumin-containing feedstock, the elution sequence includes supplementary wash steps to remove residual bound albumin. Following the post-load wash with equilibration buffer, the column is washed with pure water, then with 25 mM sodium caprylate in equilibration buffer.

In such applications, product of 70% to over 90% purity is generally obtained. A simple anion-exchange procedure can be employed to remove any remaining albumin and to provide product of approximately 98% purity. Since anion-exchange chromatography is frequently included in schemes for antibody purification, this approach is convenient and effective.

During a study in our own laboratories, IgG from a low-expressing hybridoma cell line was purified from 500-column volumes of microfiltered, protein-free cell culture supernatant. The antibody was concentrated 35-fold. Recovery of 87% was achieved in a single step, with a purity greater than 95% (*Figure 4*).

Gary Ferrell, a senior research technician at the Oklahoma Medical Research Foundation (Oklahoma City), describes his experience with HCIC. "We are using the MEP HyperCel sorbent to purify antibodies expressed using P3X63 hybridoma cells grown in LTI's hybridoma serum-free media, usually in roller bottles or flasks.

"Our final yields are about 150–175 mg of antibody per liter. HCIC has performed well in our hands. The purity achieved, greater than 95 percent, is comparable to that of our affinity-produced antibodies," Ferrell says.

"Peak elution gives optical densities in the range of 40–60 units at 280 nm. In our small-scale production lab, we can easily produce 150 mg of a monoclonal from a liter of cell culture media using a 5-ml column.

"These antibodies are clean enough for most of our applications, and it is all done in one day," Ferrell continues. "Our experience is similar with IgM-producing clones, except the capacity is about 20–25% of that for IgG."

Eszter Birck-Wilson, Ph.D., associate director of process and analytical development at **Genzyme Transgenics Corp.** (Framingham, MA), has developed a method for isolating IgG from transgenic goat milk.

Figure 4. 500 column volumes [2,000 ml of dilute feedstock (2.7 mg IgG/l)] applied to a 3.9 ml column (1 x 5 cm). SDS-PAGE of Markers (M); 2: Load (L); 3: Flow through-1 (Ft_1); 4: Flow through-2 (Ft_2); 5. Wash (W); 6: Eluate (E).

"I have been impressed with the performance of MEP Hypercel," Dr. Birck-Wilson remarks. "Protein A has been used for both analytical and preparative purification for a number of years, because it has been proven to be a powerful tool in the purification of antibodies and Fc fusion proteins.

"But the capacity and selectivity of the MEP HyperCel sorbent is at least comparable to that of protein A," she asserts. "Furthermore, the process is more economical and there is less concern about ligand leaching.

"Last but not least, in the production of injectables, it is extremely important to be able to clean and sanitize the matrix, and this matrix can withstand NaOH treatment."

Viral Clearance

Viral-clearance studies were conducted using minute virus of mouse (MVM); and a four-log reduction was achieved. The test feedstock was formulated to provide an appropriate challenge, in terms of both viral load and

protein load, and to appropriately reflect the composition of an actual cell culture supernatant.

Clarified cell culture supernatant (containing 3 mg Mab per liter) was supplemented with 200 mg murine polyclonal IgG per liter, previously purified using protein A. To this sample was added $10^{8.2}$ pfu of MVM. The eluted antibody contained $10^{4.08}$ pfu, as determined by $TCID_{50}$.

Additional Applications

The MEP HyperCel has been used in a broad range of applications, including isolation of antibodies from a variety of sources (sweet whey, colostrum, transgenic plant and transgenic animal sources) and isolation of IgA, IgM and several fusion proteins.

Gerry Bell, research scientist at **Boheringer Ingelheim Pharmaceuticals, Inc.** (Ridgefield, CT), recently reported purification of monoclonal IgE from serum-containing cell culture supernatant. IgE does not bind to protein A or protein G.

Bell evaluated the sorbent for capture directly from clarified cell culture supernatant. Preliminary studies show good separation of the IgE from impurity components, including albumin.

Thus far, the one-step HCIC purification appears comparable to Bell's usual multistep procedure (which involves ammonium sulfate precipitation, dialysis, cation-exchange and size-exclusion chromatography), but is achievable in a fraction of the time.

Various enzymes have also been successfully purified using hydrophobic charge-induction chromatography.

HCIC is thus a cost-effective alternative to traditional harvest schemes that employ protein A sorbents. Although MEP HyperCel is antibody-selective, their selectivity is sufficiently broad to provide high binding capacity for a variety of immunoglobulins.

Also, the cost of MEP HyperCel sorbent is about 25% that of comparable protein-A sorbents, while its anticipated service life is at least four times greater.

Moreover, efficient capture of product from dilute feed-stream is readily achieved; thus a column of MEP HyperCel may be viewed as a tool for both concentration and purification. These factors combine to provide substantial chemical-economic benefits. GEN

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