Large Scale Process Development for Hydrophobic Interaction Chromatography, Part 1: Gel Selection and Development of Binding Conditions

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Abstract
In hydrophobic interaction chromatography, many common causes of scale-up failure are inadvertently built into the process during the development stage. As with other adsorptive chromatographic methods, one of the most prevalent deficiencies is inadequate characterization of product retention conditions. Part 1 of this four-part series addresses gel selection and the development of sample-application conditions to avoid these problems. The authors use monoclonal antibodies to illustrate key points.

Introduction
Hydrophobic interaction chromatography (HIC) has evolved into one of the most powerful methods in preparative biochemistry. Its speed, resolution, and capacity rival ion exchange chromatography; its selectivity is complementary to other popular preparative methods such as ion exchange and size exclusion chromatography; and its ability to clear endotoxins, nucleic acids, and viruses makes it an indispensable tool for the purification of therapeutic proteins (1–8). Nevertheless, developing preparative HIC methods can be challenging. In particular, selecting a gel and developing preparative sample application conditions involve considerations that are unique to HIC. Neglecting these considerations can lead to a range of scale-up problems—including severe losses and product denaturation—that sometimes cause process developers to unnecessarily abandon this valuable technique. However, a systematic approach can avoid these problems, allowing chromatographers to develop large-scale methods that provide the same level of performance and reproducibility as other chromatographic methods.

Materials and Methods
We obtained Source 15ETH, 15ISO, and 15PHE hydrophobic interaction prepacked columns and bulk media from Pharmacia Biotech AB (Uppsala, Sweden). Ligand structures are illustrated in the accompanying box. All three media are based on 15-µm d_p monodisperse spheres with a pore-size distribution suitable for large proteins. The base matrices are composed of poly(styrene–divinylbenzene) coated with a hydrophilic polymer. The bed dimensions of the 1-mL prepacked columns are 30 mm x 6.4 mm. The bed dimensions of the 6-mL prepacked columns are 30 mm x 16 mm.

Becton Dickinson Immunocytometry Systems (San Jose, California) provided monoclonal antibodies. We used purified and unpurified

Hydrophobic Ligands

The following hydrophobic ligands were used in this study:

Source 15ETH
R-O-CH_2-CHOH-CH_2-OH

Source 15ISO
R-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2-O-CH-$(CH_3)_2$

Source 15PHE
R-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2-O-C_6H_5

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ascites samples of a mouse IgG_1 and a mouse IgM throughout this study.

We purchased buffers and salts from Sigma Chemical Company (St. Louis, Missouri). All buffer components were American Chemical Society (ACS) grade or better. Process water was prepared using reverse osmosis and deionization. We filtered buffers through a 0.22-mm filter immediately after formulation and assigned five-day expirations.

We determined the free-solution solubility of the antibodies by adding incremental concentrations of ammonium sulfate in 0.1 M sodium phosphate (pH 7.0) to purified 1-mg/mL antibody samples to create a range of final salt concentrations of 0.0–2.0 M ammonium sulfate. We incubated the samples for 1 hour at room temperature and then filtered them through a 0.22-mm filter into a twofold volume (double that of the sample) of 0.05 M sodium phosphate (pH 7.0). Then we measured each filtrate spectrophotometrically at 280 nm to determine the proportion of antibody remaining in the supernatant.

To characterize precipitation time curves we prepared duplicate sets of test tubes in advance with 1 mL of 1.50 M ammonium sulfate and 0.05 M sodium phosphate (pH 7.0). We added 100 µL of purified antibody to the first tube, allowed it to incubate for 1 min, and then filtered it to remove any precipitate. The filtrate was collected into 2 mL of 0.05 M sodium phosphate (pH 7.0) to prevent additional precipitation. We repeated the experiment with 2, 3, 4, 5, 10, 15, 30, and 60-minute incubations and quantitated the diluted filtrates spectrophotometrically. We repeated the experiment with 3.0 M ammonium sulfate, collecting the filtrates into 4 mL of diluent.

To obtain a relative expression of hydro-phobic interactions between the mouse IgG_1 and the various HIC media under nonretaining buffer conditions, we measured the peak height of the unbound material as it passed through the column. This work was performed using 1-mL prepacked columns and 20-µL injections of purified protein at 1 mg/mL in 0.05 M sodium phosphate (pH 7.0). The columns were equilibrated with 10 column volumes of ammonium sulfate with concentration ranging incrementally from 0.0 to 2.0 M. We conducted all experiments using a linear flow rate of 940 cm/hour (5 mL/min).

Using the 1-mL 15ISO columns, we determined the interfacial salt tolerance of purified proteins in a series of experiments in which we loaded protein through one line, binding buffer through another, and mixed the two streams before they reached the column. We loaded 1 mL of antibody at 5 mg/mL in 0.05 M sodium phosphate (pH 7.0) onto a 1-mL column at a mix ratio of 20% sample to 80% binding buffer (5-mL total sample-application volume). The ammonium sulfate concentration of the binding buffer varied in increments from 0.0 M to 2.5 M. In each iteration, the column was equilibrated to the mixed ammonium sulfate concentration of the intended sample stream by diluting the binding buffer on-line at a ratio of 20% 0.05 M sodium phosphate (pH 7.0) to 80% binding buffer. We determined the antibody capture efficiency for each experiment by measuring the amount of antibody recovered in the elution peak.

All experiments were conducted at room temperature, except as noted. Immunoreactivity per milligram of antibody was determined by immunoassay.

Results and Discussion

Gel selection. Selecting the most appropriate HIC medium for purification of a particular product requires careful matching of the properties of the product with those of the gel. If the ligand is too weak or at too low a density, then binding will require an excessive amount of salt. Excess salt may be a mere logistical inconvenience in some cases, but if the concentration required to accomplish binding is higher than the level at which the protein precipitates in free solution, it can severely complicate development of preparative sample-application conditions. If the ligand binds sample proteins too strongly, it can cause on-column conformational rearrangements of labile proteins. These rearrangements are sometimes reversed spontaneously upon elution, but in other cases, the protein is denatured permanently (6–13). Denaturation problems are usually accompanied by poor mass recovery, which should consequently be interpreted as a warning.

Figure 1 illustrates the results of an experiment designed to evaluate the appropriateness of 3 different supports for HIC purification of the mouse IgG_1 and the mouse IgM monoclonal antibody. For the IgG_1, we obtained mass

recoveries greater than 90% and quantitative recoveries of immunoreactivity with the 15ETH and 15ISO columns. The 15PHE support yielded a noticeable decline in both.

Mass recovery of the IgM on the 15ETH column was just less than 90% and activity recovery per milligram was quantitative. However, we encountered significant mass losses with the 15ISO column, accompanied by the loss of approximately 50% of the antibody's reactivity. The 15PHE column caused severe mass losses, accompanied by the loss of more than 75% of the immunoreactivity per mg of antibody.

Denaturation can be detected from gradient elution profiles as well as from mass and reactivity losses. Figure 2 compares the profiles of the same antibodies shown in Figure 1. With IgM on the 15ISO and 15PHE columns, the chromatograms show double-peak profiles. In each case, the protein in the second peak was visibly aggregated on elution. Although the 15ISO column appeared to allow at least diminished recovery of native protein, even the leading peak began to aggregate within 24 hours, suggesting that the entire population was affected even though only a portion of it

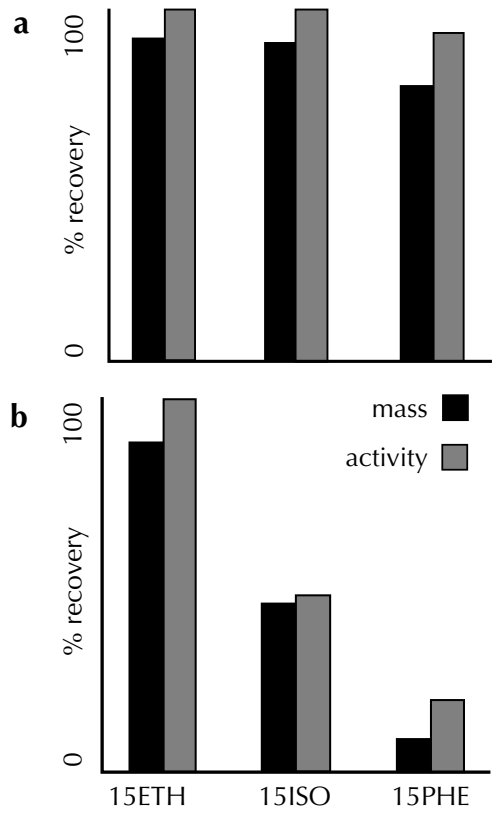

Figure 1. Recovery of (a) IgG and (b) IgM mass and activity as a function of ligand hydrophobicity. Column dimensions: 30 mm x 6.4 mm; buffer A: 2.0 M ammonium sulfate, 0.1 M sodium phosphate (pH 7.0); buffer B: 0.1 M sodium phosphate (pH 7.0); flow rate: 940 cm/hour (5 mL/min); sample: 1 mL of 5 mg/mL purified mouse IgG_1 or mouse IgM in phosphate-buffered saline; detection: UV absorbance at 280 nm, 3.0 AUFS. HIC conditions: equilibrate column with 5 mL (5 column volumes) 80% A; load sample by on-line dilution, 20% sample, 80% A; wash with 2 mL 80% A; elute antibody with a 10-mL linear gradient to 100% B; strip column with 5 mL 100% B.

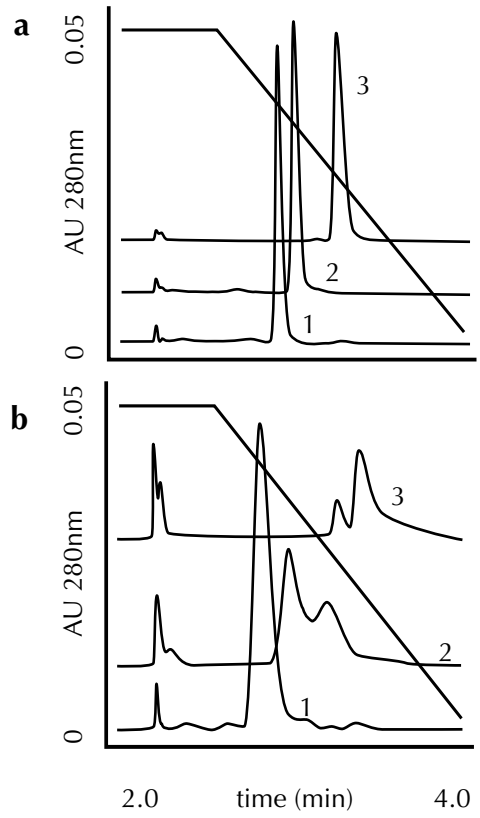

Figure 2. Gradient analyses of (a) IgG and (b) IgM using media of varying hydrophobicity: (1) 15ETH, (2) 15ISO, (3) 15PHE. Sample: 20 µL purified mouse IgG_1 or mouse IgM in phosphate-buffered saline. HIC conditions: equilibrate column with 5 mL (5 column volumes) 100% A; inject sample; wash with 2 mL 100% A; elute antibody with a 10-mL linear gradient to 100% B; strip column with 5 mL 100% B. Other conditions were the same as in Figure 1.

exhibited altered elution behavior. Process developers should thoroughly investigate any chromatographic behavior related to possible conformational rearrangements—no matter how minor—before qualifying a particular gel. The single peak from the 15ETH column showed no signs of aggregation at any time. These observations support the proposition that the best qualified gel will generally be the most hydrophobic matrix that provides high mass and activity recovery without aggregation or other conformational modifications of the product.

The best gel for purifying a given product will not always be as clearly defined as with this particular IgM. For example, although the 15ISO column provided better recoveries of the IgG_1 than the 15PHE column and was eventually selected for scale-up, the losses observed with 15PHE were not so great as to exclude it from consideration. As long as no denaturation occurs, a minor product loss at one point in purification may be offset by efficiencies elsewhere in the process.

Sample Application Conditions. Inappropriate sample application conditions are the most frequent cause of scale-up failure in HIC. As with ion-exchange chromatography, these failures usually result from underestimating the retention requirements of the product. In this context, we will define *retention* as occuring when the product remains quantitatively bound to the column for the entire duration of preparative sample loading and washing, and is eluted at the appropriate point in the gradient. Any interaction that fails to fulfill these requirements we will refer to as *retardation*.

Figure 3 compares analytical and preparative profiles of the mouse IgG_1 using the isopropyl column loaded with identical salt concentrations but different sample and wash volumes. The important difference between the two profiles is that the analytical profile suggests that the antibody is fully retained, but the preparative results reveal that it is only retarded. The sample mass (7.5 mg) was determined separately to be well within the capacity of the gel when loaded at a retaining concentration of binding salt. This type of scale-up failure arises from inadequate modeling of the volume and duration of the preparative sample load at the analytical scale. In the course of a short wash following the injection of a few microliters of sample, as shown, a product may be migrating slowly down the column, but the gradient catches up with it before it is eluted, thereby obscuring the fact that the protein is retarded

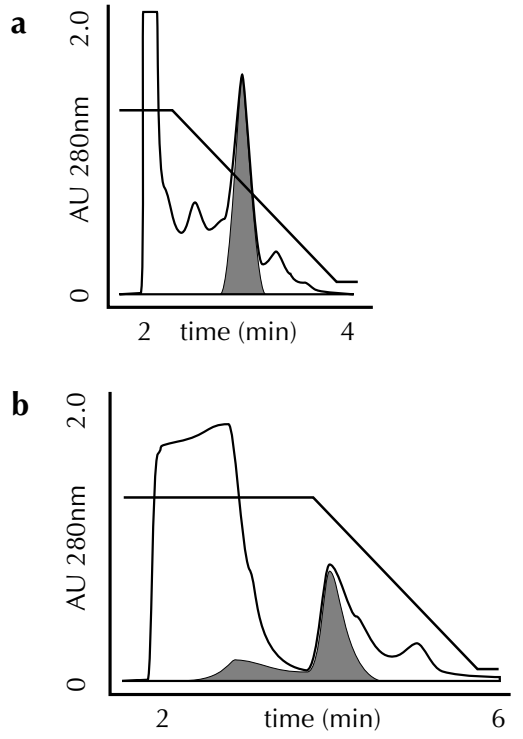

Figure 3. Scale-up failure resulting from inadequate binding conditions. Shown are (a) analytical and (b) preparative models. Column: 30 mm x 6.4 mm Source 15ISO; buffer A: 1.5 M ammonium sulfate, 0.1 M sodium phosphate (pH 7.0); buffer B: 0.1 M sodium phosphate (pH 7.0); flow rate: 940 cm/hour (5 mL/min); analytical sample: 50 mL mouse IgG_1 ascites in phosphate-buffered saline; preparative sample: 2.5 mL mouse IgG_1 ascites diluted with 2.5 mL 2.0 M ammonium sulfate immediately before injection. Analytical conditions: equilibrate column with 5 mL (5 column volumes) 66% A, 34% B; inject sample (50 mL); wash with 2 mL 66% A, 34% B; elute antibody with a 10-mL linear gradient to 100% B; strip column with 10 mL 100% B. Preparative conditions: equilibrate column with 5 mL (5 column volumes) 66% A, 34% B; apply sample (5 mL); wash with 5 mL 66% A, 34% B; elute antibody with a 10-mL linear gradient to 100% B; strip column with 10 mL 100% B. Hatched areas indicate elution position of the antibody. The mass of product loaded in the preparative experiment was approximately 7.5 mg.

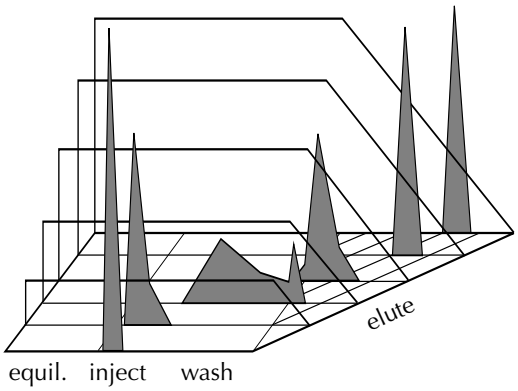

Figure 4. Diagram of hypothetical results obtained from a series of runs in which a HIC column is equilibrated with incrementally increasing concentrations of binding salt. Relative salt concentration is indicated by the height of the horizontal segment of the gradient trace.

—not retained. During extended preparative sample loading, the product may migrate enough that it begins to exit the column and continues to do so in the wash. The remainder elutes earlier than expected in the gradient. This is one of the causes of a phenomenon referred to as the *split-peak effect* (14).

Process developers can avoid this problem by characterizing retention using a series of runs in which the column is equilibrated with incrementally increased concentrations of binding salt. It is important to mimic preparative sample application by using an extended postinjection wash at the loading salt concentration. Without an estimate of how much time the eventual preparative sample application and wash intervals may require, use a wash volume of 20 column volumes as a default. Limiting injection volumes to <2% of the column volume makes it unnecessary to preequilibrate samples. Maintaining gradient slope by adjusting gradient volume facilitates direct comparison of different equilibration conditions. Results will vary significantly according to the hydrophobicity of the gel, the protein, and the choice of binding buffer.

Figure 4 is a diagrammatic representation of the type of results obtained by such an experiment. In the first profile, the protein is not even retarded. No interaction between the protein and the ligand is apparent. In the next three profiles, the protein is increas-ingly retarded,

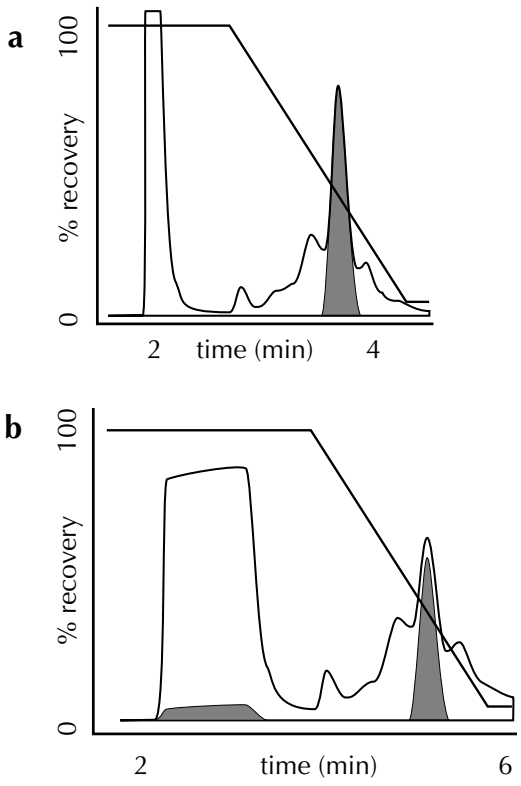

Figure 5. Scale-up failure resulting from excessive salt in the binding buffer. Column: 30 mm x 6.4 mm Source 15ISO; buffer A: 1.5 M ammonium sulfate, 0.1 M sodium phosphate (pH 7.0); buffer B: 0.1 M sodium phosphate (pH 7.0); flow rate: 940 cm/hour(5 mL/min); analytical sample: 50 mL mouse IgG_1 ascites in phosphate-buffered saline; preparative sample: 2.5 mL mouse IgG_1 ascites diluted with 2.5 mL 3.0 M ammonium sulfate immediately before loading. Analytical conditions (a): equilibrate column with 5 mL (5 column volumes) 100% A; inject sample (50 mL); wash with 2 mL 100% A; elute antibody with a 10-mL linear gradient to 100% B; strip column with 10 mL 100% B. Preparative modeling condition (b)s: equilibrate column with 5 mL (5 column volumes) 100% A; apply sample (5 mL); wash with 5 mL 100% A; elute antibody with a 10-mL linear gradient to 100% B; strip column with 10 mL 100% B. Hatched areas indicate elution position of the antibody.

but the interaction is not strong enough to keep it bound. In the last two profiles, the protein is fully retained. The next-to-last profile indicates the minimum retention requirements with respect to binding salt concentration.

Although experiments using purified product provide useful preliminary estimates of retention requirements, the retention thresholds they reveal may be altered by the final process conditions. Three additional factors must be considered. The first is the composition of the intended sample. If the product of interest is the strongest binding component of the mixture, then retention conditions should conform closely to those of pure-protein experiments. If the sample contains large amounts of contaminants that bind more strongly than the product, then displacement may occur and pure-protein experiments may underestimate the concentration of binding salt required for retention. The second factor is the binding capacity of the gel at the intended linear flow rate. If the gel is loaded beyond capacity, product losses are inevitable despite otherwise appropriate binding conditions. Like selectivity, capacity is affected significantly by sample composition. (Capacity is discussed in part 3 of this series.) The third factor is the combined volume of the sample and preelution wash. Process developers should study the effects of all these variables thoroughly before proceeding to scale-up.

Scale-up failure frequently results from neglecting to consider the solubility characteristics of the sample. Figure 5 illustrates another example of the split-peak effect; in this case, caused by excessive salt in the binding buffer. The unbound fractions in the preparative profile were slightly hazy after collection, became cloudy within 1–2 min, and developed obvious precipitates within 10–15 min. We isolated the precipitates using centrifugation, resuspended them, and determined that the chief component was antibody. After increasing the concentration of salt in the binding buffer in an attempt to improve retention, the problem became worse. This combination of circumstances suggested that the column failed to retain the unbound fraction of antibody because aggregates formed that were large enough to be excluded by the beads but still small enough to pass between them.

As in Figure 3, this phenomenon usually fails to occur with sample injections smaller than a few percent of the column volume. Presumably, the precolumn residence time of the sample in the binding buffer is too brief for significant aggregate populations to develop, and precipitates are present at a level too low for casual detection.

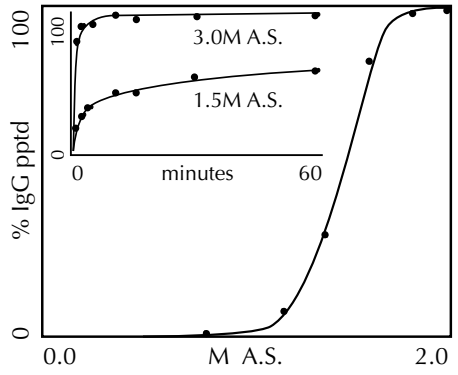

Figure 6. Precipitation curve of a mouse IgG_1 monoclonal antibody as a function of ammonium sulfate concentration. Experimental conditions are described in the text.

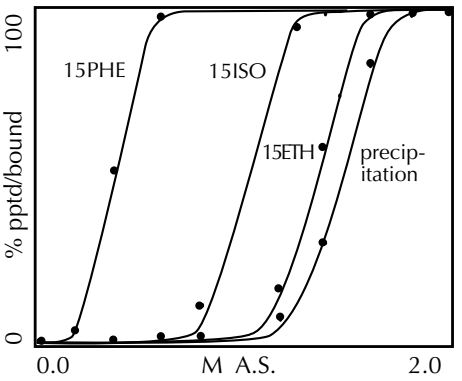

Figure 7. Hydrophobic interaction curves of a mouse IgG_1 monoclonal antibody with various HIC media, compared with the precipitation curve of the same antibody. Experimentals conditions are described in the text.

Figure 6 illustrates the precipitation curve of the mouse IgG_1 monoclonal antibody as a function of ammonium sulfate concentration. The inset shows the time precipitation curve for the same antibody at 1.50 M and 3.0 M ammonium sulfate. We derived two important conclusions from the results: First, precipitation occurs within a wide range of salt concentrations, beginning at levels substantially below those necessary for quantitative precipitation. Second, precipitation is strongly time dependent. Figure 7 illustrates the interaction curves of the same antibody obtained using the

15ETH, 15ISO, and 15PHE columns, compared with the antibody's precipitation profile. These data reveal the source of many scale-up problems and help to define the conditions for avoiding them.

At the salt concentration required to accomplish complete product retention using the 15ISO columns (~1.35 M), the antibody in free solution is approximately 25% precipitated after 1 hour. Preparative-scale batch loading under such conditions invariably leads to scale-up failure similar to that illustrated in Figure 5. Often it is more severe because of column blockage by precipitates.

How could a process developer set a sample-loading specification that would permit such a problem? The answer resides in the time dependency of precipitation. At the limited process scale under which method development often takes place, the volumes of material are small and easier to handle, and operations move quickly from step to step. These circumstances allow the early stages of aggregation to be overlooked, especially if the sample is relatively crude. Minor product losses are often dismissed as tolerable. Even if aggregation or precipitation of the product does not result in scale-up failure, it likely will result in a poorly reproducible manufacturing process. Variation with respect to how long in advance the sample is prepared and in the time it takes to load are inevitable at full process scale.

The situation when using the 15PHE column is fundamentally different. This particular antibody was fully retained at 0.75 M ammonium sulfate, well beneath its precipitation threshold. Assuming no problems with product recovery or denaturation, this product could be batch equilibrated in advance and loaded without compromising process performance or reproducibility. As a general rule, the stronger the ligand a protein can endure, the more permissive the sample loading conditions.

This solution does not address the problem of how to conduct preparative sample loading of proteins that are too labile to withstand strongly hydrophobic supports but require salt concentrations within their precipitation ranges to support retention on weaker supports. The answer, again, resides in the time dependency of precipitation. With chromatography systems configured to support gradient applications, samples can be loaded through a dedicated

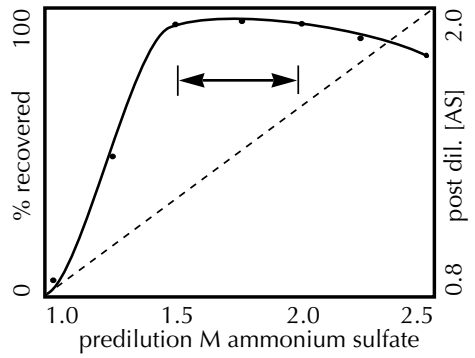

Figure 8. Interfacial salt tolerance of a mouse IgG_1 monoclonal antibody during binding by on-line dilution. The binding window is indicated by the arrow. Experimental conditions are described in the text.

line and mixed on-line with a binding-buffer concentrate to yield a mixture that has the average salt concentration necessary to ensure retention. This technique is called *continuous on-line dilution* (15).

With on-line dilution, the sample's precolumn residence time is reduced to the range of a few seconds or minutes, depending on the flow rate and the internal fluid volumes of the chromatography system. This residence time allows the proteins to enter the column environment and be adsorbed before aggregation or precipitation can become problematic. The other defining characteristic—and an important advantage compared with off-line batch equilibration—is that the exposure time of sample to salt is invariant. This supports much better reproducibility than can be obtained with a batch approach.

As with any dilution technique intended for large-scale application, users have a strong motivation to keep process volumes as low as possible by minimizing the dilution factor. This would normally translate into using the highest possible salt concentration in the binding buffer. However, this approach is usually disastrous with HIC. Precipitation accelerates proportionately with increasing salt, occurring almost instantaneously if the salt concentration is high enough (see Figure 6). During on-line dilution, this will cause the product to precipitate as it contacts the salt interface. This can occur even if the average salt concentration in

the postdilution mix is less than the product's precipitation point. This phenomenon occurs because the protein has insufficient time to re-equilibrate after precipitation at the binding buffer–sample interface.

Figure 8 illustrates the results of a set of experiments designed to identify the interfacial salt tolerance of the IgG_1 antibody (see "Materials and Methods" section). When the predilution binding-buffer salt concentration is less than approximately 2.0 M ammonium sulfate, the retention curve of the protein on the 15ISO column is roughly the same as our earlier estimate (Figure 7). This indicates that the protein is able to tolerate the salt concentration at the interface without precipitation.

At levels higher than 2.0 M ammonium sulfate, interfacial aggregation results in reduced capture efficiency, which yields a split-peak effect similar to that illustrated in Figure 6 and can lead to severe back-pressure problems. These data define an on-line dilution binding window for this antibody of: ammonium sulfate as high as 2.0M in the binding buffer, at a mix ratio of not more than 40% sample to 60% binding buffer; or ammonium sulfate as low as 1.4M in the binding buffer, with a mix ratio of not more than 10% sample to 90% binding buffer. Because the leading boundary of the window is defined by the hydrophobicity of the gel and the trailing boundary is defined by the protein's solubility characteristics, the window will always be broader for more strongly hydrophobic gels and narrower for weaker ones.

Figure 9 illustrates a preliminary scale-up run of the IgG_1 on a 90-mL (5 cm x 5 cm) 15ISO column with 1.5 M ammonium sulfate in the binding buffer at a mix ratio of 20% ascites to 80% binding buffer. We estimated the single-step purity of the indicated fraction as approximately 80%. Mass recovery was 92% (762 mg), and recovery of immunoreactivity was quantitative.

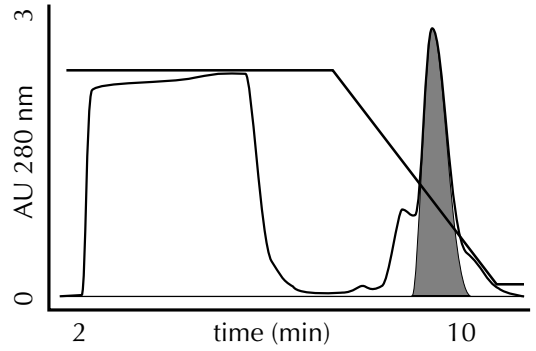

Figure 9. Preliminary HIC scale-up isolation of mouse IgG_1 monoclonal antibody. Column: 5 cm x 5 cm Source 15ISO; buffer A: 1.5 M ammonium sulfate, 0.1 M sodium phosphate (pH 7.0); buffer B: 0.1 M sodium phosphate; flow rate: 750 cm/hour (250 mL/min); sample: 175 mL of clarified mouse IgG_1 ascites, filtered through a 0.22-mm filter. Run conditions: equilibrate column with 450 mL (5 column volumes) 80% A, 20% B; apply sample by on-line dilution, 80% A, 20% ascites; wash with 450 mL 80% A, 20% B; elute antibody with a 900-mL linear gradient to 100% B; strip column with 900 mL 100% B. Hatched area indicates elution position of the antibody.

Conclusions

The development of successful preparative HIC processes depends on careful attention to a variety of factors that are common to most preparative chromatography methods. These factors include sample preparation, the development of fractionation conditions, and the determinations of flow rates and capacity. HIC requires that process developers also consider some variables that are usually taken for granted in other techniques, chiefly including the potential for product denaturation on strongly hydrophobic surfaces and potential product insolubility under binding conditions. In HIC, these two factors dominate gel selection and the development of sample-application conditions. This makes them primary determinants of successful scale-up.

Thorough gel evaluation and development of sample application conditions can support effective HIC methods for even the most labile proteins, regardless of process scale. This undeniably involves more development work than other methods but, with this extra effort, HIC can provide the same performance, reproducibility, and process economy as large-scale ion-exchange processes. Given the technique's unique selectivity and its ability to enhance the effectiveness of other methods, the investment is unquestionably worthwhile.

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